EFFECT OF VASCULAR HETEROGENEITY, AGING, AND EXERCISE TRAINING ON eNOS – ASSOCIATED PROTEIN:PROTEIN INTERACTIONS

A Dissertation

by

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ABSTRACT

Endothelial dysfunction is a major risk factor for the development of cardiovascular diseases, and aging is associated with a gradual decline in endothelial function. Furthermore, endothelial dysfunction in arteries and arterioles supplying skeletal muscle has been implicated in the decline in exercise capacity with aging. Defined as an imbalance between the production and degradation of nitric oxide (NO), limited NO bioavailability is the hallmark characteristic of endothelial dysfunction. Production of NO is controlled by the enzyme endothelial nitric oxide synthase (eNOS), which is regulated in part by post-translational protein modifications.

The purpose of this research was to examine the effect of vascular heterogeneity, aging, and endurance exercise training on eNOS-associated protein:protein interactions. Caveolin-1 (Cav1) is a negative regulator of eNOS activity, so that NO production is suppressed when Cav1 is bound to eNOS. Conversely, calmodulin (CaM) and heat-shock protein 90 (Hsp90) are positive regulators of eNOS activity, thus increasing eNOS activity and NO production when either are bound to eNOS. Co-immunoprecipitation was used to determine protein:protein interactions among eNOS and its regulatory proteins throughout the arterial network, from the aorta to third order skeletal muscle arterioles. Results show that eNOS-associated protein:protein interactions vary throughout the arterial network, and Cav1:eNOS and CaM:eNOS interactions are altered with aging. Additionally, endurance exercise training has no effect on the protein:protein interactions examined. In conclusion, eNOS regulation via protein:protein interactions

appears to be vessel-specific, and aging has a heterogeneous effect on protein:protein interactions throughout the arterial network.

This work is dedicated to my lovely parents, Jeff and Sueda, for their unconditional love and support. It's a blessing to be your daughter.

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INTRODUCTION

1.1 Identification of endothelium-derived relaxing factor as nitric oxide

The vascular endothelium is composed of a monolayer of cells lining the inner wall of blood vessels separating vascular smooth muscle from the circulating blood (Luescher and Barton 1997). This layer of cells was traditionally believed to be an inert physical barrier until groundbreaking experiments by Furchgott and Zawadzki demonstrated an active role of the endothelium in maintaining vascular homeostasis (Furchgott and Zawadzki 1980). In this seminal study, rabbit aortic rings that had their endothelial cells removed were exposed to acetylcholine (ACh), which resulted in the development of tension through the action of ACh on the muscarinic receptors on vascular smooth muscle. However, when care was taken to maintain the endothelium intact, ACh acted via the endothelium through a then unknown mechanism to cause relaxation of the aortic rings. From these experiments, Furchgott and Zawadzki concluded that the endothelium was capable of producing a substance that causes relaxation of the surrounding vascular smooth muscle in response to ACh stimulation of the endothelium. This substance was termed "endothelium-derived relaxing factor" (EDRF). Expanding on Furchgott and Zawadzki's discovery, this substance was later identified to be nitric oxide (NO) (Palmer et al. 1987; Ignarro et al. 1987a). Additional research has identified a number of EDRF's that are now known to play a role in mediating endothelium-dependent vasodilation, including cyclooxygenase (COX)-derived prostacyclin (PGI_2) (Holtz et al. 1984), and endothelium-derived hyperpolarizing

factor (EDHF) (Brandes et al. 2000). Furthermore, studies have also demonstrated a role of the endothelium in producing vasoconstrictors, including endothelin-1(Boulanger and Luscher 1990; Lüscher et al. 1990). Collectively, these studies were among the first to establish the role of the vascular endothelium as a paracrine organ, able to respond to chemical and mechanical stimuli by producing and releasing vasoactive substances to cause local vasodilation or vasoconstriction. This enables blood flow to the perfused tissue to be precisely regulated in order to match oxygen delivery to metabolic demand, maintaining local vascular homeostasis.

1.2 Roles of nitric oxide in vascular function

Since the confirmation of NO as an EDRF, several other physiological roles of NO have been identified. In addition to regulating vascular tone NO also helps prevent the development of atherosclerotic lesions, and aids in the process of new blood vessel growth, all in order to maintain vascular homeostasis (Murohara et al. 1998; Hogg et al. 1993). Human studies have established the importance of NO bioavailability in maintaining endothelial function and cardiovascular health. Impaired NO-mediated endothelial function has been demonstrated with aging and in pathological conditions including obesity, hypertension, insulin resistance, and high cholesterol (Higashi et al. 2001; Heitzer et al. 2000; Casino et al. 1993). These conditions are all risk factors for developing cardiovascular diseases, with impaired NO bioavailability a potential link between the pathology of the original disease and subsequent cardiovascular complications (Li and Förstermann 2000). These studies demonstrate the clinical importance of NO as a regulator of vascular function and healthy endothelial function.

Vascular tone is maintained by the balance of vasodilators and vasoconstrictors produced and present in the vasculature. Of these, NO is a key endothelium-derived vasodilator that acts on the adjacent vascular smooth muscle to increase intracellular cyclic GMP levels. Subsequently, reduced intracellular calcium causes the vascular smooth muscle to relax, resulting in vasodilation (Ignarro et al. 1987b). Endothelial nitric oxide synthase (eNOS) is a calcium-dependent enzyme responsible for catalyzing the conversion of L-arginine to NO and L-citrulline (Palmer et al. 1988; Ignarro 1990). In addition to L-arginine, numerous cofactors, including tetrahydrobiopterin (BH_4), NADPH, FAD, and FMN are also required for this reaction to occur (Michel and Feron 1997). NO-mediated, endothelium-dependent vasodilation has been demonstrated in response to a number of physiological agonists, including ACh, vascular endothelial growth factor (VEGF), bradykinin, and histamine. While the intracellular signaling pathways for each agonist are distinct, all result in an increase in the intracellular calcium concentration in endothelial cells.

Endothelium-dependent, NO-mediated vasodilation can also be induced by physical forces acting on the wall of blood vessels. Shear stress created by blood flowing across the luminal surface of endothelial cells is an important mechanical signal controlling local vasodilation (Koller et al. 1993). Additionally, shear stress is a key mechanism responsible for stimulating NO release in response to increases in blood flow. Shear stress has been shown to cause calcium-dependent NO synthesis in endothelial cells (Xiao et al. 1997). This is of particular interest in the study of blood flow regulation during exercise, as the increase in blood flow to actively contracting

skeletal muscles causes local vasodilation, allowing a greater oxygen and nutrient delivery to actively contracting skeletal muscles during exercise.

There is also evidence suggesting that pulsatile changes in intraluminal pressure on vessel walls can induce endothelium-mediated NO release. This effect was originally demonstrated by Awolesi et al. (1994) who demonstrated that increasing cyclic strain on the surface of the endothelial cells increased eNOS activity and NO production in cultured cells. Following this finding, Recchia et al. (1996) demonstrated that acute increases in intraluminal pulsatile pressure *in vivo* enhanced endothelium-derived NO release in dog coronary arteries. While this mechanism of regulating endothelial NO production remains to be fully examined, results of these studies suggest that acute increases in intraluminal pressure may be an important mechanism accounting for the hyperemic response to exercise, and promote healthy endothelial function.

Nitric oxide is generally considered to be an athero-protective molecule, meaning that its bioavailability can prevent or mitigate the development of atherosclerosis and subsequent cardiovascular disease. This effect of NO is due, in part, to its anti-adhesion effects on platelets and leukocytes (Tsao et al. 1994; Freedman et al. 1997) and inhibitory effects on smooth muscle cell proliferation (Garg and Hassid 1989), which is an early step in the development of atherosclerosis. The presence of NO in the vascular endothelium is critical to prevent the accumulation of platelets and leukocytes in the wall of blood vessels at the site of endothelial injury, whereas impaired NO-mediated endothelial function is an early event in the pathogenesis of atherosclerosis (Reddy et al. 1994; Zeiher et al. 1991b). Evidence from Hogg et al. (1993) also demonstrate a role of

NO in inhibiting low density lipoprotein (LDL) aggregation and oxidation and uptake by macrophages *in vitro*. In addition to these effects, NO also inhibits the proliferation of vascular smooth muscle at the site of vascular injury (Janssens et al. 1998). These studies show that the presence of NO in the endothelium protects against the pathogenesis of atherosclerosis by preventing the accumulation of platelets, leukocytes, and LDL cholesterol that leads to the development of the plaque in the wall of the vessel that is characteristic of atherosclerosis. Importantly, these studies emphasize the protective effects of NO against the development of cardiovascular diseases.

Finally, NO plays an important signaling role in angiogenesis in response to tissue ischemia and the activation of vascular endothelial growth factor (VEGF) (Cooke 2003; Murohara et al. 1998). The process of angiogenesis is stimulated by local tissue ischemia in an effort to maintain blood flow and oxygen delivery to the ischemic tissue. VEGF is thought to be the primary stimulus secreted by ischemic tissue that results in endothelial NO production (Papapetropoulos et al. 1997). VEGF acts on tyrosine kinase receptors on the surface of endothelial cells and activates the intracellular PI3K/Akt second messenger cascade (Achen et al. 1998; Dimmeler et al. 2000). As a result, Akt phosphorylates eNOS on serine residue 1177, an activating site on the eNOS protein (Dimmeler et al. 2000). Supporting evidence for these findings include a study by Zicher et al. (Ziche et al. 1997) who showed that inhibition of eNOS activity with L-NG-nitroarginine methyl ester (L-NAME) inhibited angiogenesis in rabbit corneal vessels. Furthermore, in eNOS knockout mice, VEGF-induced angiogenesis was impaired compared to wild-type mice, confirming the role of eNOS and NO in VEGF-induced

angiogenesis (Fukumura et al. 2001). These studies establish the critical role of eNOS-derived NO production in initiating tissue angiogenesis as a mechanism to restore adequate blood flow and nutrient delivery to ischemic tissue or to increase skeletal muscle angiogenesis as an adaptation to exercise training.

1.3 Regulation of nitric oxide bioavailability

Nitric oxide bioavailability is determined by the balance between NO production and NO degradation. Proper balance of NO produced versus NO degraded is critical in maintaining local vascular homeostasis and ensuring adequate tissue perfusion so that oxygen and nutrient delivery is precisely matched to oxygen and nutrient demand of the perfused tissue. An imbalance between the production and degradation of NO leads to endothelial dysfunction, a condition which increases the risk of developing cardiovascular diseases (Halcox et al. 2002; Celermajer et al. 1994a). The bioavailability of NO is regulated by a number of physiological factors, including the presence or absence of enzyme substrates and cofactors, endothelial cell agonists, and mechanical stimuli, which act in concert to maintain vascular endothelial homeostasis (**Figure 1.1**).

Figure 1.1

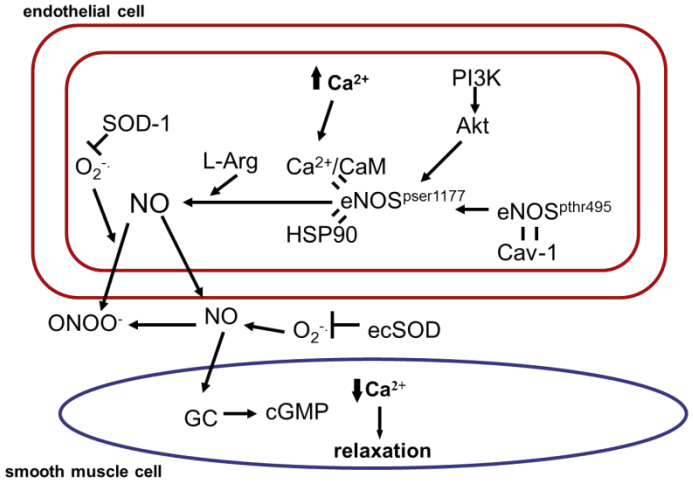

Figure 1.1. Mechanisms regulating nitric oxide bioavailability.

As mentioned in the previous sections, endothelial cell agonists such as ACh and VEGF increase eNOS enzyme activity and NO production. Additionally, mechanical stimuli such as shear stress and intraluminal pulsatile pressure also increase eNOS enzyme activity and NO production, improving local vascular homeostasis (Woodman et al. 2005; Woodman et al. 2007). The mechanical stimulation of eNOS activity may be an important mechanism accounting for the hyperemic response to exercise in active skeletal muscles, which will be discussed in detail in a later section.

NO degradation can occur via several different mechanisms. However, these mechanisms may interact with each other and in pathological conditions, may exacerbate each other. For example, oxidative stress in the vascular endothelium may deplete tetrahydrobiopterin (BH_4), an important co-factor for eNOS enzyme activity (Alp and Channon 2004; Bendall et al. 2005). This in turn results in "eNOS uncoupling", meaning

that eNOS becomes an oxidant-producing enzyme, further contributing to the oxidative stress in the endothelium (Landmesser et al. 2003).

NO is rapidly scavenged by reactive oxygen species (ROS) present in the vasculature. ROS are oxygen-containing ions that are present in nearly all tissues. Because they carry a negative charge, ROS are highly reactive and rapidly combine with other molecules in oxidation-reduction reactions where the original ROS is reduced and another molecule, such as NO, is oxidized (Beckman et al. 1990). Additionally, ROS may also have endothelium-dependent and independent vasoconstrictive effects, although this effect does not appear to be true of all ROS or in all vascular beds. Trott et al. (2011) have demonstrated endothelium-dependent vasorelaxation in response to the ROS hydrogen peroxide (H_2O_2) in soleus feed arteries from young and old rats. The vasoactive properties of H_2O_2 appear to vary with the vascular bed, as it causes vasodilation in porcine coronary arteries (Barlow and White 1998), rat mesenteric arteries (Barlow and White 1998), and rat skeletal muscle arteries and arterioles (Trott et al. 2011; Sindler et al. 2009), while causing vasoconstriction in porcine coronary arterioles (Thengchaisri et al. 2006). However, care should be taken when interpreting these findings as there may be a species effect on the role of H_2O_2 in vasoregulation.

Superoxide anion ($O_2^{\cdot -}$) is another ROS that is a product of cellular respiration, making it ubiquitous in the body. NAD(P)H oxidase and xanthine oxidase are two of the primary enzymes responsible for the production of $O_2^{\cdot -}$ in the endothelium, although NADPH oxidase appears to be primarily responsible for elevated ROS (Donato et al. 2007). When $O_2^{\cdot -}$ reacts with NO, peroxynitrite ($ONOO^-$) is produced (Beckman et al.

1990). Under normal conditions in healthy endothelium, levels of ROS are maintained by antioxidant enzymes including superoxide dismutase, and catalase (Beckman et al. 1988). However, in many pathological conditions, ROS concentrations exceed the ability of antioxidants to scavenge the ROS, leading to tissue damage (Guzik et al. 2000). ROS in the endothelium has been shown to be elevated with aging (Trott et al. 2011), smoking (Tanriverdi et al. 2006), and disease states (Heitzer et al. 2001; Ceriello et al. 2002). Consequently, the presence of excess ROS is one mechanism that has been extensively examined as a mechanism resulting in NO-dependent endothelial dysfunction in these conditions.

In addition to oxidative stress, in the absence of adequate levels of its co-factor BH_4, eNOS is also capable of producing O_2^{-}, further contributing to oxidative stress in the surrounding tissue and limiting NO bioavailability (Alp and Channon 2004; Cosentino and Lüscher 1999). This process, known as eNOS uncoupling, has been shown to be another possible mechanism of NO-dependent endothelial dysfunction with aging and Type II diabetes (Heitzer et al. 2000). Indeed, increasing the availability of BH_4 *in vivo* improves NO-mediated endothelial function in both animals and humans (Eskurza et al. 2005). The rate-limiting step in the production of BH_4 is the enzyme guanosine triphosphate-cyclohydrolase (GTP-CH). *In vitro* studies suggest that increasing GTP-CH gene expression increases BH_4 production, suggesting another possible mechanism for limited BH_4 bioavailability. In fact, extensive work by Alp and colleagues has demonstrated a therapeutic effect of GTP-CH gene overexpression on

NO-mediated endothelial function in atherosclerosis and diabetes (Alp et al. 2004; Alp et al. 2003).

In addition to the availability of BH_4, the presence of the substrate for eNOS, L-arginine, has also been implicated in limiting NO bioavailability in the endothelium (Rees et al. 1989). Several studies have shown a beneficial effect of L-arginine supplementation on NO-mediated endothelial function in individuals with cardiovascular diseases (Lerman et al. 1998; Hambrecht et al. 2000a; Drexler et al. 1991). It is hypothesized that in conditions of impaired NO bioavailability supplementation with L-arginine may increase NO production to compensate for the rapid NO degradation by ROS (Hambrecht et al. 2000a). Additionally, L-arginine may also act as an antioxidant so that NO is preserved in pathological conditions (Huk et al. 1997; Böger et al. 1998).

Post-translational modifications to the eNOS protein also play a role in maintaining NO bioavailability. Phosphorylation of eNOS can either enhance NO or suppress NO production, depending on which residue is phosphorylated (Mount et al. 2007). A study by Trott et al. (Trott et al. 2013) demonstrated a link between eNOS phosphorylation on the enzyme activating residue Ser^{1177} and NO-mediated endothelial function with ACh in young and old soleus feed arteries. In contrast, there have been fewer studies examining the effect on NO bioavailability of phosphorylation of eNOS on its negative regulatory residue, Thr^{495}. While Thr^{495} has been shown to suppress eNOS enzyme activity *in vitro* (Fleming et al. 2001), to our knowledge this finding has yet to be confirmed *in vivo*. Regardless, these studies suggest that post-translational eNOS phosphorylation is a critical mechanism for maintaining NO bioavailability.

Another post-translational mechanism regulating eNOS enzyme activity is its interaction with other intracellular proteins. In endothelial cells, eNOS activity is regulated by caveolin-1 (Cav1), a negative regulator of eNOS activity (Du and Chen 2009; Smith et al. 2006; Michel et al. 1997a), as well as heat-shock protein-90 (Hsp90), and calmodulin (CaM), positive regulators of eNOS activity (Garcia-Cardena et al. 1998; Michel et al. 1997a). Conditions that result in enhanced eNOS interaction with Cav1 and/or reduced interactions with Hsp90 and CaM may reduce NO bioavailability. A role of protein:protein interactions between eNOS and its regulatory proteins have been demonstrated in vitro. Additionally, in vivo studies have linked an increase in Cav1:eNOS interaction with reduced NO bioavailability and impaired endothelial function with aging. Furthermore, there is some evidence that exercise training enhances Hsp90:eNOS activity in skeletal muscle (Harris et al. 2008); however the implications for NO bioavailability and NO-mediated vascular endothelial function remain to be determined.

Maintaining NO bioavailability is a critical mechanism for maintaining vascular homeostasis. Conditions resulting in reduced NO bioavailability and impaired NO-mediated endothelial function increase an individual's risk for developing cardiovascular disease independent of the presence of any other cardiovascular risk factors.

1.4 Clinical implications of nitric oxide deficiency

Cardiovascular diseases are a leading cause of death among adults in the United States, accounting for one third of all deaths in the year 2009 (Go et al. 2013). Additionally, healthcare and lost-productivity costs attributable to cardiovascular

diseases are estimated to be $312.6 billion per year (Go et al. 2013). Endothelial dysfunction is an independent risk factor for developing cardiovascular disease, and occurs early in the process of cardiovascular disease development (Celermajer et al. 1994a). Aging is also an independent risk factor for cardiovascular disease, and is associated with a decline in vascular endothelial function, further compounding the risk of developing cardiovascular disease with age (Celermajer et al. 1994c). Endothelial damage is the initiating step in the process of atherosclerosis (Ross and Glomset 1976; Ludmer et al. 1986) and endothelial dysfunction is present even when there is no other clinical evidence of atherosclerotic lesions (Celermajer et al. 1994a). The age-related decline in endothelial function has been demonstrated in various vascular beds in older humans and aged animal models (Küng and Lüscher 1995; Muller-Delp et al. 2002a; Cernadas et al. 1998; Kang et al. 2009a; Celermajer et al. 1994c; van der Loo et al. 2000a; Trott et al. 2013; Trott et al. 2011; Woodman et al. 2003). Similar to the decline in endothelial function, there is also an age-related decline in aerobic capacity and exercise tolerance (Fleg et al. 2005; Fitzgerald et al. 1997). Lack of physical activity throughout the lifespan may account for the reduced exercise tolerance with aging; furthermore lifelong physical inactivity may also account for the age-related impairment in NO-mediated endothelial function. The presence of endothelial dysfunction throughout the arterial vascular tree is not only a risk factor for cardiovascular disease, but is also a potential mechanism accounting for the impaired endurance exercise tolerance seen with aging. In large elastic conduit arteries like the aorta and brachial artery, endothelial dysfunction is linked to the development of atherosclerosis and

cardiovascular disease risk (Mullen et al. 2001; Woo et al. 1997). Downstream of the conduit arteries, endothelial dysfunction in skeletal muscle resistance arteries and arterioles may result in impaired exercise hyperemia, potentially reducing oxygen delivery to actively contracting skeletal muscles and limiting maximal exercise capacity (Musch et al. 2004). However, endurance exercise training has been shown to attenuate the effects of aging on endothelial function and cardiovascular disease risk while also improving endothelial function in skeletal muscle resistance vessels (Eskurza et al. 2004; DeSouza et al. 2000; Hambrecht et al. 2000b; Trott et al. 2009; Spier et al. 2004). Thus the beneficial effects of endurance exercise training have systemic effects on vascular endothelium, and specifically improve cardiovascular risk factors and exercise tolerance.

In pathological conditions including cardiovascular diseases, Type I and II diabetes, smoking, hyperlipidemia, hypertension, and aging, the balance between NO production and degradation is disrupted so that NO-mediated vasodilator responses to stimuli are blunted. This endothelial dysfunction compounds the original condition by increasing the risk of developing cardiovascular complications, further contributing to the healthcare costs associated with cardiovascular diseases.

1.5 Mechanisms of nitric oxide deficiency

Impaired endothelial function can be attributed in part to reduced NO bioavailability, the mechanisms of which have been discussed in a previous section. NO deficiency can result from insufficient NO production via eNOS, increased NO degradation by oxidative stress, or a combination of mechanisms suppressing NO production and increasing NO degradation. This may be due to a number of factors,

including lack of substrate or cofactors for eNOS, reduced eNOS protein content, and/or post-translational modifications of eNOS that suppress its enzyme activity.

In a study conducted by van Haperen et al, (van Haperen et al. 2002) eNOS overexpression resulted in reductions in blood pressure and markers of atherosclerosis in apo-E deficient mice, suggesting that restoring eNOS function and NO bioavailability improves cardiovascular risk factors. However, as will be discussed in more detail in the following section, in some conditions, eNOS protein content may be elevated compared to healthy endothelium, possibly as a compensatory mechanism to maintain endothelium-dependent NO production in the presence of impaired NO signaling. Additionally, NO degradation by ROS is elevated in many pathological conditions, likely due to an increase in ROS generation and a decrease in the antioxidant capacity of the endothelium.

Interventions that increase antioxidant availability, like ascorbic acid infusion, have been shown to improve NO-mediated endothelial function (Eskurza et al. 2004). Because endothelial dysfunction has been implicated in several diseases and conditions, it is likely that the mechanisms accounting for NO deficiency may vary with the disease or condition being investigated. Additionally, the mechanisms of NO deficiency throughout the arterial tree may be vessel-specific. The vascular heterogeneity of the vasoactive response to H_2O_2 has already been discussed in a previous section. Changes in eNOS protein content with age also appear to be vessel specific (Woodman et al. 2002). Results of these studies support the concept of the heterogeneous nature of vascular endothelial function and dysfunction, and that other mechanisms of impaired

NO bioavailability may follow a similar pattern. This heterogeneous phenotype of impaired NO bioavailability adds a layer of complexity to the study of the mechanisms of endothelial dysfunction.

1.6 Endothelium, nitric oxide and aging

Endothelial function declines with age, thus increasing the risk of cardiovascular diseases with advancing age (Celermajer et al. 1994a). As a consequence, cardiovascular diseases are the leading cause of mortality in adults over the age of 65 (Gorina Y 2006). Impaired NO-mediated endothelial function with aging has been demonstrated in various vascular beds in both humans and animal models of aging. The mechanisms accounting for impaired NO-mediated endothelial function with age appear to be varied as well. Furthermore, the implications for age-associated endothelial dysfunction are multi-faceted. For example, age-related endothelial dysfunction in resistance-sized arteries feeding skeletal muscles likely contributes to reduced exercise tolerance in older adults (Woodman et al. 2002; DeSouza et al. 2000). As a result, this may contribute to the established age-related decline in maximal aerobic capacity (Wilson and Tanaka 2000; Fitzgerald et al. 1997). The importance of maintaining aerobic capacity with age has been demonstrated by Sui et al. (2007), who showed a negative correlation between aerobic capacity and all-cause mortality in adults aged 60 and over. Additionally, maintaining aerobic capacity with aging may allow older adults to maintain an independent lifestyle until very late in life.

In large elastic conduit arteries like the brachial artery, impaired endothelial function is linked to the development of atherosclerosis and arterial stiffening with age

(Taddei et al. 1995). As outlined in a previous section, endothelial dysfunction occurs early in the process of atherosclerosis. The development of endothelial dysfunction and subsequent atherosclerosis increase the likelihood of having a serious cardiovascular event, potentially resulting in significant healthcare costs and loss of independence in older adults.

1.7 Mechanisms of age-induced endothelial dysfunction

Several mechanisms accounting for impaired NO bioavailability in the endothelium have been discussed in section 1.3. Many of these same mechanisms have been investigated in order to determine the cause(s) of the age-related decline in NO-mediated endothelial function (**Table 1.1**).

One potential mechanism accounting for the age-related decline in endothelial function is reduced eNOS protein transcription and translation. However, results from previous studies examining eNOS protein content with aging show inconsistent results, with some studies showing an increase, (Goettsch et al. 2001; van der Loo et al. 2000a) others showing a decrease, (Barton et al. 1997a; Tanabe et al. 2003a; Woodman et al. 2005; Csiszar et al. 2002) and others showing no change in eNOS protein content (Woodman et al. 2002). Sindler et al. (Sindler et al. 2009) demonstrated that endothelium-dependent dilation is impaired with aging in rat skeletal muscle arterioles, despite an increase in eNOS protein content. The discrepancy of results may reflect differences in the vascular bed being studied, as additional research has demonstrated that endothelium-dependent dilation is preserved with aging in arteries feeding glycolytic skeletal muscle, while in feed arteries perfusing oxidative skeletal muscles,

endothelium-dependent dilation is impaired with age, despite no age differences in total eNOS content in either glycolytic or oxidative skeletal muscle feed arteries (Woodman et al. 2002).

Another mechanism hypothesized to account for the age-related decline in NO-mediated endothelial function is the well-documented increase in oxidative stress seen with aging. Scavenging of NO by pro-oxidants reduces NO bioavailability even when eNOS protein content and enzyme activity are unchanged. Trott et al. (Trott et al. 2011) have demonstrated that inhibition of a key oxidant enzyme, NAD(P)H oxidase, restored NO-mediated endothelial function in soleus muscle feed arteries (SFA). Furthermore, this same study also showed that $O_2^{\cdot-}$ scavenging with TEMPOL and H_2O_2 scavenging with catalase restored NO-mediated endothelial function. Elevated content of NAD(P)H oxidase subunit gp91phox was also found in the aged SFA, which may explain the elevated oxidative stress seen with age.

The availability of eNOS cofactors, particularly BH_4 is another potential mechanism that may account for the decline in endothelial function with aging. Delp et al. (Delp et al. 2008) have demonstrated that there is a reduction in BH_4 content with age in soleus muscle first-order arterioles. Without BH_4, eNOS becomes a pro-oxidant enzyme, thus reducing NO bioavailability by 1) decreasing the production of NO from eNOS, and 2) increasing the oxidative stress in the local environment that scavenges NO that is produced (Bendall et al. 2005).

Table 1.1: Mechanisms of Age-Related Impairments in Nitric Oxide Bioavailability

Citation	Model	Age	Vessel	Vascular responses with age	Mechanism Investigated
Cernadas, 1998.	Wistar	young: 5 mo; old 18 mo	aorta	↓ responsiveness to ACh, BK	↑eNOS and iNOS content
Csiszar, 2002.	Sprague-Dawley	young: 14 weeks; old: 80 weeks	coronary arterioles	↓ FID; augmented with SOD and Tiron supplementation	↓eNOS mRNA, ↑iNOS mRNA expression
Delp, 2008.	Fisher 344	young: 6 mo; old: 24 mo	S1A	↓FID; no effect of arginase inhibition or L-arginine; sepiapterin ↑ FID	↓BH_4 content
Sindler, 2009.	Fisher 344	young: 3 mo; old: 22 mo	S1A	↓FID	↑eNOS-derived O_2^- in old S1A; ↓BH_4 and flow-induced NO bioavailability with age
Smith, 2006.	Fisher 344 x Brown Norway	young: 6 mo; old: 36 mo	aorta	↓ endothelium-dependent, ACh-induced vasorelaxation	no change in eNOS protein content with age; ↓membrane-bound eNOS with age; ↑ Cav1:eNOS, ↓ Hsp90:eNOS, ↓Akt:eNOS with age
Soucy, 2006.	Wistar	young: 3-4 mo; old: 22-24 mo	aorta	↓ ACh-induced vasorelaxation ; ↓shear stress induced NO release ; ↑ PWV	↓ p/t - Akt ratio with age; ↓ p/t - eNOS ratio with age
Spier, 2004.	Fisher 344	young: 4-7 mo; old: 24-26 mo	S1A, G1A	↓ACh- ID in S1A only	in S1A: no age effect on eNOS mRNA but ↑eNOS content; in G1A: no age effect on eNOS mRNA or protein content
Trott, 2013.	Fisher 344	young: 4 mo; old: 24 mo	SFA	↓FID and ACh- ID; Akt and PI3K inhibition abolished age group differences	↓ flow- and ACh-induced p-eNOS on Ser1177 with age; PI3K inhibition abolishes age group differences in p-eNOS ser1177

Table 1.1 continued

Citation	Model	Age	Vessel	Vascular responses with age	Mechanism Investigated
van der Loo, 2000.	Fisher 344 x Brown Norway	young: 4-6 mo; middle: 19 mo; old: 32-35 mo	aorta	↓ ACh-induced vasorelaxation in old vs young and middle age; ↓ endothelium-dependent relaxation to A23187	↓A23187-induced NO release with age; ↑ eNOS protein content and activity with age; ↑A23186 - stimulated O_2^- release with age
Woodman, 2002.	Fisher 344	young 4 mo; old: 24 mo	SFA, GFA	↓ ACh- ID in SFA only	↓ eNOS and SOD-1 protein content with age in SFA only; no change in eNOS or SOD-1 mRNA expression with age in either SFA or GFA
Woodman, 2003.	Fisher 344	young 4 mo; old: 24 mo	SFA	↓ FID and ACh- ID	↓ NO-mediated flow- and ACh-induced dilation
Woodman, 2005	Fisher 344	young 4 mo; old: 24 mo	SFA	↓ ACh- ID; improved with acute flow treatment	↓ eNOS mRNA expression with age; ↑ eNOS mRNA expression with age in response to flow treatment
Woodman, 2007.	Fisher 344	young 4 mo; old: 24 mo	SFA	↓ FID and ACh- ID; improved with acute high pressure treatment	pressure treatment ↑ eNOS mRNA expression in young but not old SFA; no change in eNOS protein content with pressure treatment in either old or young SFA

Abbreviations: BK – bradykinin: iNOS – inducible nitric oxide synthase; FID – flow-induced dilation; SOD – superoxide dismutase; S1A – soleus first order arterioles; BH_4 – tetrahydrobiopterin; NO – nitric oxide; PWV – pulse wave velocity; G1A – gastrocnemius first order arterioles; ACh – ID – acetylcholine-induced dilation; SFA - soleus feed arteries; GFA – gastrocnemius feed arteries

Changes in post-translational mechanisms like protein:protein interactions that modulate eNOS activity have not been thoroughly explored in the context of aging. eNOS activity is regulated by a number of proteins, including Cav1, a negative regulator of eNOS activity (Du and Chen 2009; Smith et al. 2006; Michel et al. 1997a), and Hsp90 and CaM, positive regulators of eNOS activity (Garcia-Cardena et al. 1998; Michel et al. 1997a). Recent evidence from endothelial cell culture studies suggests that eNOS-associated protein:protein interactions are altered with aging (Smith et al. 2006) which may explain the reduced NO bioavailability seen in aged arteries. An increase in oxidative stress has been shown to increase eNOS association with Cav1 as a protective mechanism against oxidant activity of eNOS, or eNOS uncoupling *in vitro* (Karuppiah et al. 2011). eNOS uncoupling has been demonstrated in aged skeletal muscle feed arteries (Sindler et al. 2009); however, whether eNOS uncoupling is a mechanism accounting for altered protein:protein interactions with aging remains to be determined. Additionally, to our knowledge, no studies have examined protein:protein interactions among eNOS and Hsp90 or CaM, representing an gap in the current knowledge in both the aging and endothelial physiology literature.

1.8 Endothelium, nitric oxide and exercise training

Aerobic exercise training improves endothelial function in both healthy individuals (Clarkson et al. 1999) and people with diseases or conditions that impair endothelial function (DeSouza et al. 2000; Graham and Rush 2004; Hambrecht et al. 2000b). In several studies, this improvement has been attributed to improved NO bioavailability (Graham and Rush 2004; Green et al. 2004). Additionally, improvements

in endothelial function with exercise training have been shown in several vascular beds, including rat aorta (Graham and Rush 2004), rat skeletal muscle feed arteries and arterioles (Trott et al. 2009; Koller et al. 1995). In human studies, the brachial artery (Graham and Rush 2004) and coronary arteries (Hambrecht et al. 2000b) have demonstrated improvements in endothelial function with exercise training. These studies demonstrate a systemic effect of endurance exercise training on endothelial function; however, the mechanism(s) accounting for this improvement is not fully understood. Aerobic exercise results in numerous changes within the cardiovascular system that may modulate endothelial function both acutely and chronically. It is likely that these endothelial adaptations to aerobic exercise training confer much of the protective cardiovascular benefits of exercise.

During acute bouts of exercise, the role NO-mediated endothelial function in mediating exercise hyperemia in exercising muscle remains unclear. Methodological inconsistencies in studies examining NO-mediated endothelium-dependent vasodilation in exercising skeletal muscle make extrapolating the conclusions from these studies difficult. For example, Wilson et al. (1993) demonstrated that pharmacological inhibition of eNOS immediately prior to exercise did not significantly decrease limb muscle blood flow with exercise. In contrast, Dyke et al. (1995) showed that eNOS inhibition during skeletal muscle contractions attenuated blood flow to the exercising limb. However, in this same study NO inhibition blunted limb blood flow by approximately the same magnitude at rest as during exercise. This may mean that NO helps maintain vascular tone both at rest and during exercise, but it may not be solely responsible for the increase

in blood flow with exercise. Following up on these studies, Schrage et al. (Schrage et al. 2004) showed that inhibition of eNOS and PGI_2 during exercise reduced hyperemia in the exercising limb, although the contribution of PGI_2 to forearm hyperemia was less than that of NO. Collectively, these studies suggest that NO contributes to exercise hyperemia in contracting muscles; however, there may be redundant mechanisms controlling this phenomenon. Therefore, NO may contribute but may not be obligatory to the increase in skeletal muscle blood flow during exercise in young, healthy subjects.

Chronic endurance exercise training improves NO-mediated endothelial function at rest, which improves the maintenance of vascular homeostasis and importantly, cardiovascular disease risk. An early study by Sessa et al. (Sessa et al. 1994) showed that eNOS gene expression and NO production are upregulated in the coronary arteries of exercise-trained dogs, and hypothesized that the mechanism accounting for this was the increase in shear stress during a single bout of exercise. Shear stress across the luminal surface of endothelial cells has also been identified as a primary stimulus that initiates adaptations in the endothelium resulting in an increased NO bioavailability with regular aerobic exercise in humans (Tinken et al. 2009; Tinken et al. 2010). Chronic intermittent elevations in shear stress with exercise training initiates many adaptations that improve endothelial function long-term.

Intermittent increases in intraluminal pressure during exercise may also confer benefits on endothelial function. Woodman et al. (Woodman et al. 2007) tested the effects of short-term increases in intraluminal pressure, mimicking the increase in intraluminal pressure during an acute bout of exercise, on NO-mediated endothelial

function in young and old SFA. Interestingly, they found that while acute treatment with elevated pressure improved NO-mediated, flow-induced vasodilation in old SFA, the improvement in endothelial function was not accompanied by an increase in eNOS mRNA expression. However, in young SFA, short-term pressure treatment increased both eNOS and SOD-1 mRNA expression, but had no effect on NO-mediated endothelial function. Thus, it appears that short-term increases in intraluminal pressure may improve NO bioavailability in old SFA; however, the mechanism accounting for the improvements in NO-mediated endothelial function in old SFA with short-term increases in intraluminal pressure remains to be determined.

Chronic endurance exercise training also appears to improve the cellular signaling mechanisms responsible for maintaining NO bioavailability. One potential beneficial effect of endurance exercise training is the reduction in oxidative stress, which may be due to an upregulation of antioxidant enzymes like SOD-1 (Rush et al. 2003) or downregulation of oxidant enzymes like NAD(P)H subunit gp91phox (Graham and Rush 2004). This in turn reduces the scavenging of NO by ROS, increasing its bioavailability and improving NO-mediated endothelial function.

Post-translational mechanisms regulating eNOS may also be altered with exercise training, resulting in improved NO-mediated endothelial function. Hambrecht et al. (Hambrecht et al. 2003) found improved NO-mediated endothelial function accompanied by increased eNOS phosphorylation on Ser^{1177} in coronary arteries from individuals with coronary artery disease who were exercise trained for 4 weeks. The increase in eNOS phosphorylation on Ser^{1177} was correlated to an increase Akt

phosphorylation, suggesting that exercise training favorably alters Akt pathway signaling in the endothelium resulting in improved NO synthesis via eNOS.

In vitro research has demonstrated an important role of shear stress in regulating Cav1 interactions with eNOS (Rizzo et al. 1998b), suggesting that protein:protein interactions may be regulated by the mechanical stimuli associated with exercise, and may thus be altered by exercise training. There is also evidence suggesting that exercise training increases the interaction between Hsp90 and eNOS (Harris et al. 2008), although this was found in the soleus muscle tissue, not vascular endothelial cells. However, the possibility remains that the same or similar mechanisms that increase Hsp90:eNOS interaction in skeletal muscle may also alter Hsp90:eNOS interaction in the endothelium. Regardless, more research is needed to thoroughly investigate this mechanism of post-translational regulation of eNOS with exercise training.

Finally, endurance exercise appears to attenuate or reverse the negative effects of aging on endothelial function (Trott et al. 2009; Spier et al. 2004; Fitzgerald et al. 1997; DeSouza et al. 2000). In a cross-sectional study, Master's athletes (64.7 ± 1.4 y/o) who had engaged in lifelong aerobic exercise had greater endothelium-dependent dilation in the brachial artery compared to age-matched, sedentary individuals. In a separate study (Taddei et al. 2000), endurance-trained elderly men (66.4 ± 6.1 y/o) had endothelium-dependent forearm vasodilator responses greater than their sedentary counterparts (62.9 ± 6.1 y/o) and similar to young sedentary men (26.9 ± 2.3 y/o). Additionally, previously sedentary older adults who started an endurance exercise program improved brachial artery endothelial function compared to their counterparts who remained sedentary, and

restored endothelial function to levels similar to the young sedentary men (DeSouza et al. 2000). The beneficial effects of exercise training on NO-mediated endothelial function have been demonstrated in animal models of aging as well (Spier et al. 2004; Trott et al. 2009; Hashimoto 1990). Results from these exercise training studies in animal models further support the beneficial effects of endurance exercise training on endothelial function with aging.

1.9 Regional heterogeneity of endothelial cell aging

In the arterial tree, there are key anatomical differences between the large conduit arteries, and smaller resistance-sized arteries and arterioles. These anatomical differences reflect the different functional requirements of the different types of arteries. In large conduit arteries like the aorta and brachial, iliac, and femoral arteries, there is a thick medial elastic layer allowing the high pressure generated by the heart to be absorbed and stored as potential energy in what is described as a "Windkessel" effect. The recoil of the elastic tissue in conduit arteries keeps blood flowing through the cardiovascular system during diastole of the heart. In humans, conduit artery endothelial function can be assessed using ultrasonography using brachial artery flow-mediated dilation (Corretti et al. 2002). Endothelial function in conduit arteries declines with age, and is associated with the development of atherosclerosis and the development of cardiovascular diseases (Celermajer et al. 1994a). Along with the decline in endothelial function with age, there is also a loss of arterial compliance, and an increase in arterial stiffness, exacerbating the risk of developing cardiovascular diseases and hypertension (Tanaka et al. 2000). A cross-sectional study of individuals with and without coronary

heart disease established a significant positive correlation between NO-mediated endothelial function and conduit artery stiffness (Nigam et al. 2003). However, no causal link between endothelial function and arterial stiffness was established in this study. Endothelium-derived NO contributes to the maintenance of basal tone in conduit vessels (Fok et al. 2012; Stewart et al. 2003; Fitch et al. 2001), and NO inhibition increases measures of arterial stiffness during changes in blood flow (Bellien et al. 2010) in healthy adults.

In a study conducted by Soucy et al. (2006) a potential mechanism linking endothelium-derived NO and aortic stiffness in aging was explored. In this study, old rats had impaired NO-mediated endothelial function, reduced NO bioavailability, and increased pulse-wave velocity (PWV; a measure of aortic stiffness) compared to young rats. Furthermore, eNOS inhibition impaired aortic stiffness and endothelial function in the young, while exogenous NO improved aortic stiffness and endothelial function in the old aortas. Finally, this study also identified impaired PI3K/Akt signaling in the old aortas as one potential mechanism for the increase in aortic stiffness with age. These studies highlight the role of endothelium-derived NO in the function of conduit arteries and establish a link between the aging process and impaired endothelial function in conduit arteries. Despite the documented link between endothelium-derived NO and the maintenance of vascular tone, the mechanisms accounting for the age-related loss of NO-mediated endothelial function in conduit arteries remain to be determined.

In contrast to the large conduit arteries, resistance sized arteries and arterioles have multiple layers of smooth muscle in the medial layer regulating the diameter of the

vessel and allowing for fine tuning of regional blood flow to precisely meet oxygen and nutrient demand with blood supply. While endothelial dysfunction in large elastic conduit arteries is linked to the development of cardiovascular diseases, endothelial dysfunction in the smaller, resistance sized arteries and arterioles is linked to altered blood flow in a variety of tissue (Prisby et al. 2007; Zeiher et al. 1991a). Interestingly, aging has been shown to impair skeletal muscle blood flow with moderate intensity exercise to oxidative skeletal muscles, whereas blood flow is elevated in arteries perfusing glycolytic skeletal muscles (Musch et al. 2004). The altered blood flow pattern between oxidative and glycolytic skeletal muscle is hypothesized to be one mechanism accounting for the age-related decline in exercise capacity (Irion et al. 1987). Research by Woodman et al. (Woodman et al. 2002) also demonstrated muscle fiber-type differences in NO-mediated endothelial dysfunction, showing that endothelial function is maintained with age in gastrocnemius feed arteries (GFA), while endothelial function is impaired in SFA. This finding is supported by a later study by Hirai et al. (Hirai et al. 2011) who showed that both vascular conductance (an indicator of blood flow) and NO-dependent endothelial function is impaired with age in SFA compared to GFA. Together, these results suggest that aging exerts differential effects on endothelial phenotype in resistance arteries from glycolytic vs. oxidative skeletal muscles that account for the regional differences in blood flow and endothelial function.

Additionally, there appears to be heterogeneity in the effects of aging on the arterioles downstream of the feed arteries in both oxidative and glycolytic muscles. Muller-Delp et al. (Muller-Delp et al. 2002) found that while flow-induced, NO-

mediated vasodilation was impaired with age in both soleus and gastrocnemius first order arterioles, ACh-induced vasodilation was only impaired in first order arterioles from the soleus muscle. This study also demonstrates regional heterogeneity within the rat hindlimb with respect to endothelial phenotype and function with aging. However, the mechanisms accounting for the differences in alterations in endothelial function between oxidative and glycolytic resistance-sized vessels are not fully understood.

The mechanisms underlying the age-associated impairment in NO-mediated endothelial function remain to be fully elucidated. Furthermore, the apparent regional heterogeneity of the effects of age on endothelial function adds an additional layer of complexity to the understanding of these mechanisms. It is likely that the decline in NO-mediated endothelial function with aging is related to disparate changes in the local environment between conduit and resistance arteries, and between arteries and arterioles perfusing oxidative vs. glycolytic skeletal muscles. As a result, it is important to distinguish the various mechanisms accounting for the age-related decline in endothelial function in different vascular beds so that appropriate countermeasures can be developed that target specific mechanisms of aging in specific vessels to achieve individualized outcomes.

1.10 Hypotheses and purpose

To our knowledge, this is the first set of studies examining the effects of age and aerobic exercise training on protein:protein interactions regulating eNOS activity in aged arteries and arterioles throughout the arterial tree. Additionally, these studies help establish a phenotype of protein:protein interactions among eNOS and its regulatory

proteins in endothelium of young and old arteries of both conduit and resistance-sized vessels. The hypotheses of the following studies are: 1) aging results in an impairment of NO-mediated endothelium-dependent vasorelaxation in the rat aorta; 2) the impaired endothelium-dependent vasorelaxation in aged rat aortas is associated with alterations in protein:protein interactions between eNOS and its regulatory proteins (specifically, Cav1, Hsp90, and CaM); 3) endurance exercise training improves or restores NO-mediated vasorelaxation in aged aortas by reversing the detrimental effects of aging on protein:protein interactions between eNOS and its regulatory proteins; 4) the endothelial phenotype of protein:protein interactions between eNOS and its regulatory proteins will not be uniform throughout the arterial tree, from the aorta through low-order skeletal muscle arterioles; 5) aging will have a heterogeneous effect on the endothelial phenotype of protein:protein interactions between eNOS and its regulatory proteins throughout the arterial tree, from the aorta through low-order skeletal muscle arterioles; and finally, 6) there will be a difference in endothelial phenotype of protein:protein interactions between eNOS and its regulatory proteins with age and skeletal muscle fiber type between soleus muscle and gastrocnemius muscle feed arteries and arterioles.

The purpose of the research presented in this dissertation is to set the groundwork for a comprehensive investigation of a poorly understood mechanism of impaired endothelial function with age. Consequently, it is expected that the impact of this research to be a step forward in understanding of the effects of aging and the beneficial effects of exercise on NO-mediated vascular function throughout the arterial tree. This contribution will be significant because this project will focus on an unexplored

mechanism of age-related endothelial dysfunction that may be of significant interest in the development of therapeutic strategies to reduce the impact of cardiovascular diseases prevalent in older individuals. Furthermore, identifying a mechanism of endothelial dysfunction that is also a site of adaptation to aerobic exercise may lead to the development of cost-effective strategies for prevention and management of age-related endothelial dysfunction and consequent cardiovascular diseases, thus improving the quality of life for older adults.

CHAPTER II

EFFECT OF AGING ON PROTEIN:PROTEIN INTERACTIONS AMONG eNOS AND KEY REGULATORY PROTEINS THROUGHOUT THE ARTERIAL NETWORK

2.1 Introduction

An age-related decline in nitric oxide (NO)-mediated endothelial function has been demonstrated in numerous vascular beds, resulting in an increased risk of developing cardiovascular diseases (Celermajer et al. 1994a). Due to the heterogeneity of structure and function throughout the arterial tree, the effects of aging on NO-mediated endothelial function are similarly varied. Regional heterogeneity in endothelial function with age has been demonstrated in skeletal muscle feed arteries from oxidative versus glycolytic skeletal muscle (Woodman et al. 2002). In this study, aging resulted in an impaired endothelium-dependent, NO-mediated dilation in soleus feed arteries (SFA), while endothelial function in gastrocnemius feed arteries was preserved. Resistance-sized arteries and arterioles are sensitive to metabolic regulation of vascular tone, and it is possible that the metabolic milieu of the glycolytic portion of the gastrocnemius versus the oxidative soleus muscle accounts for difference in the effect of age on NO-mediated endothelial function. The disparate effect of aging on endothelial function in glycolytic versus oxidative skeletal muscle feed arteries is consistent with alterations in skeletal muscle blood flow to glycolytic and oxidative skeletal muscles with age. As has been previously shown by Musch et al. (2004), there is a reduced ability to increase blood flow to oxidative skeletal muscle and an enhanced blood flow to glycolytic

skeletal muscle during exercise with age. Thus, it is likely that differences in blood flow patterns and the local environment during exercise contribute to regional differences in vascular endothelial function. Furthermore, in some large arteries like the femoral artery, NO-mediated endothelial function is also preserved with age, while endothelial function in the aorta declines (Barton et al. 1997c). The authors of this study attribute these differences to regional differences in pulse pressure stimulus between the aorta and femoral artery. These studies suggest the possibility that the mechanisms accounting for the decline in NO-mediated endothelial function with age may vary throughout the vascular tree.

Age-related changes in post-translational mechanisms regulating eNOS enzyme activity are an intriguing candidate as a potential mechanism accounting for the impaired NO bioavailability with age. One post-translational change that may impair NO-mediated endothelial function with age is alterations in protein:protein interactions among endothelial nitric oxide synthase (eNOS) and its key regulatory proteins, caveolin-1 (Cav1), and calmodulin (CaM). Cav1 is a negative regulator of eNOS activity (Michel et al. 1997a), so that an increase in Cav1 binding to eNOS may limit NO bioavailability and result in impaired NO-mediated endothelial function. In contrast, CaM is a positive regulator of eNOS activity (Michel et al. 1997a; Garcia-Cardena et al. 1998), thus potentially increasing enzyme activity and NO bioavailability when bound to eNOS. Work by Smith et al. (2006) demonstrated reduced Cav1:eNOS interaction with age in aortas; however, the significance of changes in protein:protein interactions with age throughout the vascular tree remains unknown.

The purpose of the present study was to: 1) characterize the phenotype of eNOS-associated protein:protein interactions throughout the vascular tree, from the abdominal aorta to third order skeletal muscle arterioles; 2) determine the effect of aging on the phenotype of eNOS-associated protein:protein interactions throughout the vascular tree; and 3) compare the effect of aging on the phenotype of eNOS-associated protein:protein interactions between the feed arteries and arterioles in glycolytic versus oxidative skeletal muscles.

2.2 Methods

2.2.1 Animals

Prior to the start of this study, approval for the use of animals was obtained from the University of Missouri and Texas A&M University's Institutional Animal Care and Use Committees. Young (4 mo, n = 9) and old (24 mo, n = 9) male Fischer 344 rats were obtained from a commercial dealer (Harlan Sprague-Dawley, Indianapolis, IN) and housed at the University of Missouri College of Veterinary Medicine's Animal Care Facility. All rats were housed under a 12:12-h light-dark cycle and food and water were provided *ad libitum.* The rats were examined daily by the Animal Care Facility veterinarians or staff.

2.2.2 Isolation of vascular tissue

Prior to the removal of vascular tissue, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 – 60 mg/kg body wt, ip). The thoracic and abdominal cavities were exposed and sections of the abdominal aorta, iliac, and femoral arteries were dissected out, placed in 0.7 uL microcentrifuge tubes, snap

frozen, and stored at -80°C for subsequent co-immunoprecipitation and immunoblot analysis.

To collect the skeletal muscle feed arteries and arterioles, the gastrocnemius/soleus skeletal muscle complex was removed from the hindlimbs and placed in cold (4°C) MOPS buffered physiological saline solution (PSS), containing (in mM): 145.0 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.17 $MgSO_4$, 1.2 NaH_2PO_4, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA and 25.0 MOPS (pH 7.4). The gastrocnemius/soleus complex was then transferred to a Lucite chamber containing MOPS-PSS for dissection of skeletal muscle feed arteries and arterioles. The soleus muscle feed artery (SFA), first order (S1A), second order (S2A), and third order (S3A) arterioles were each dissected out and placed in separate 0.7 mL microcentrifuge tubes, snap frozen, and stored at -80°C for subsequent co-immunoprecipitation and immunoblot analysis. The gastrocnemius muscle was separated into the white (glycolytic) portion and red (oxidative) portions. The gastrocnemius feed artery (GFA) and first order arteriole (G1A) were identified, along with the second and third order arterioles of the red portion (RG2A, RG3A, respectively) and second and third order arterioles of the white portion (WG2A, WG3A, respectively). These vessels were dissected out and also placed in separate 0.7 mL microcentrifuge tubes, snap frozen, and stored at -80°C for subsequent co-immunoprecipitation and immunoblot analysis.

2.2.3 Immunoprecipitation

The aorta, iliac arteries, and femoral arteries were homogenized using a mortar and pestle, then transferred to a microcentrifuge tube with a lysis buffer containing (in

mM): 60.0 octyl-β-d-glucopyranoside (Sigma-Aldrich, O8001), 150.0 NaCl, 20.0 Tris base, 1% protease inhibitor cocktail (EMD Millipore, 20-201), 1% phosphatase inhibitor cocktail (Sigma-Aldrich, P0044). These vessel segments were further homogenized using repeated freeze-thaw cycles with brief sonication. For the smaller vessels (SFA, GFA, S1A, G1A, RG2A, RG3A, WG2A, WG3A), lysis buffer was added to the microcentrifuge tubes for homogenization using repeated freeze-thaw cycles. 8-10 arterioles were pooled to ensure adequate protein concentration for each group. To collect the soluble proteins from all vessels, the homogenates were centrifuged for 10 min at 12,000Xg at 4°C and the supernatant collected and used for co-immunoprecipitation.

2.2.3.1 Co-Immunoprecipitation of arteries

For co-immunoprecipitation of the aorta and arteries, 20μL of Protein G/A agarose suspension was added to the lysate and incubated at 4°C for 1 hour under gentle rotary agitation. After centrifugation at 12,000XG for 1 min, the supernatant was collected. 1 μg of polyclonal eNOS antibody was added to the lysate and incubated for 4 hours at 4°C under gentle rotary agitation. 15μL of Protein G/A agarose suspension was then added to the lysate and incubated for 1 hour at 4°C under gentle rotary agitation. The eNOS protein complex was then eluted from the lysate, washed three times and stored at -80°C in Laemmli buffer for immunoblotting.

2.2.3.2 Co-Immunoprecipitation of arterioles

For co-immunoprecipitation of G1A, S1A, RG2A, RG3A, WG2A, and WG3A, 1 μg of polyclonal eNOS antibody was added to the lysate and incubated overnight at 4°C

under gentle rotary agitation. 15μL of Protein G/A agarose suspension was then added to the lysate and incubated for 1 hour at 4°C under gentle rotary agitation. The eNOS protein complex was then eluted from the lysate, washed twice and stored at -80°C in Laemmli buffer for immunoblotting.

2.2.4 Quantification of Cav1:eNOS and CaM:eNOS interactions

Relative differences in eNOS, Cav1, and CaM content between age groups were assessed in all vessels using immunoblot analysis as described in detail previously (Jasperse and Laughlin 1999). eNOS, Cav1 and CaM protein content were evaluated using monoclonal antibodies (eNOS and CaM, 1:1250; Cav1, 1:2500). Immunoblots were evaluated using enhanced chemiluminescence (ECL, Amersham) and densitometry using LAS-4000 Luminescent Image Analyzer and Multi-Gauge Image Analysis Software (FUJIFILM Medical Systems).

2.2.5 Statistical analysis

All values are presented as mean ± SE. Two-way ANOVA was used to determine age- and vessel-group differences in protein:protein interactions. One-way ANOVA was used to determine vessel-group differences in protein:protein interactions within each age group individually. Statistical significance was set at the $P \leq 0.05$ probability level. Due to the limited amount of arteriolar tissue available for biochemical analysis, some of these groups were excluded from statistical analysis due to small n-size, although the means are displayed in the graphs below.

2.3 Results

2.3.1 Total eNOS protein content

Total eNOS protein content in all vessels is shown in **Figure 2.1** Total eNOS protein content was not significantly altered with age in any of the vessels studied. There were no significant differences between any vessels in total eNOS protein content.

2.3.2 Cav1:eNOS interactions

Cav1:eNOS interaction in all vessels is shown in **Figure 2.2**. Cav1:eNOS interaction was not significantly altered with age in any of the vessels studied. There were no significant differences between any vessels in Cav1:eNOS interaction.

2.3.3 CaM:eNOS interactions

CaM:eNOS interaction in all vessels is shown in **Figure 2.3**. CaM:eNOS interaction was not altered with age. However, among young vessels, CaM:eNOS interaction was significantly greater in SFA compared to the aorta, iliac, femoral, and gastrocnemius feed artery, WG3A, and S2A.

Figure 2.1

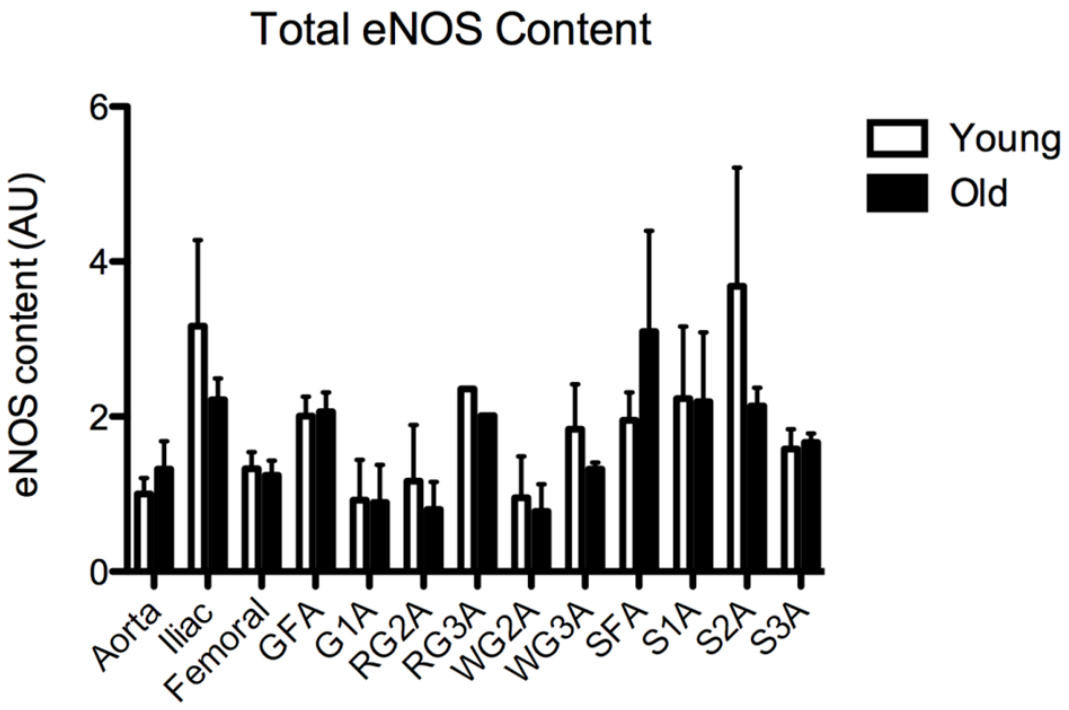

Figure 2.1. Total eNOS protein content in arteries and arterioles. Values are means ± SE; No significant differences between groups. Values with no standard error bars have n ≤ 2 per group.

Figure 2.2

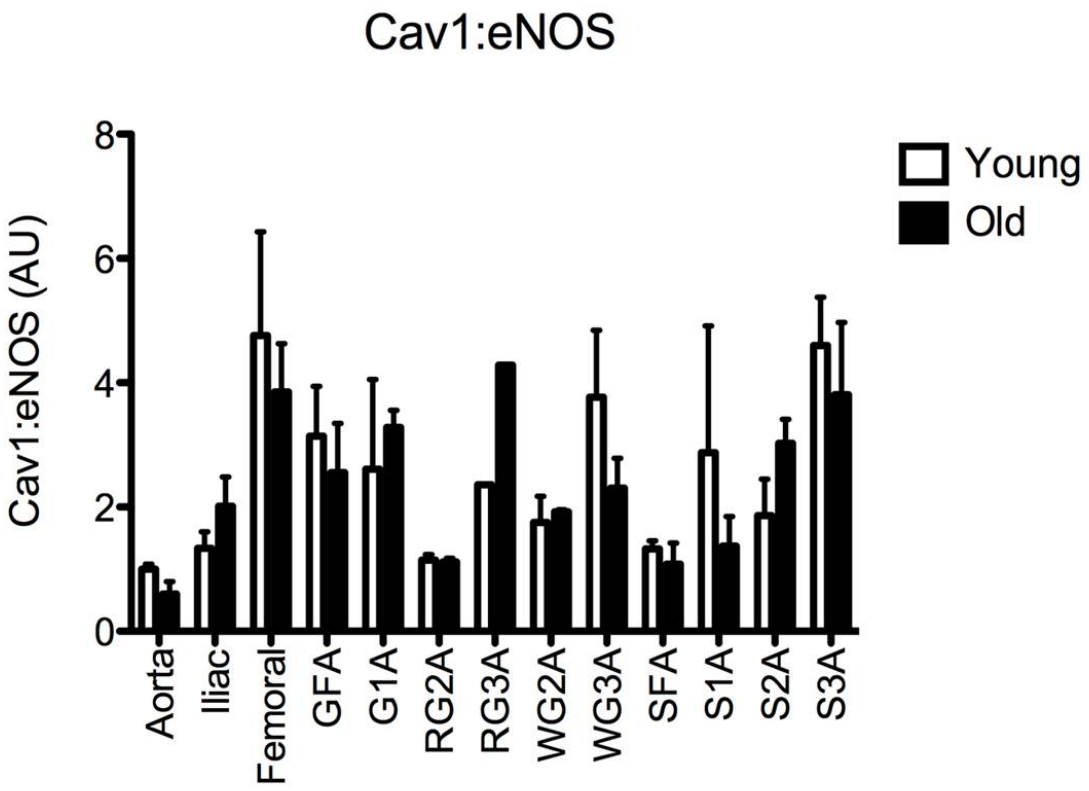

Figure 2.2. Vascular heterogeneity of Cav1 association with eNOS. Antibodies against eNOS were used to immunoprecipitate eNOS protein. The immunoprecipitates were analyzed for associated Cav-1 by immunoblot. No significant differences between groups. Values with no standard error bars have n ≤ 2 per group.

Figure 2.3

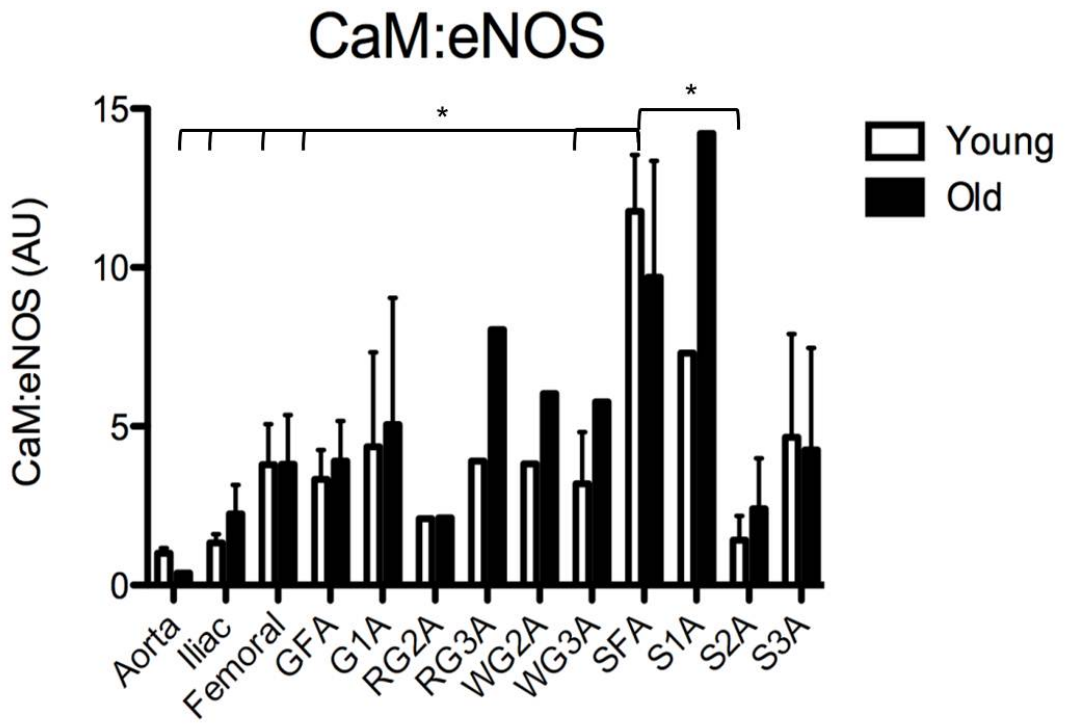

Figure 2.3. Vascular heterogeneity of CaM association with eNOS. Antibodies against eNOS were used to immunoprecipitate eNOS protein. The immunoprecipitates were analyzed for associated CaM by immunoblot. Values are means ± SE. n = 3-6/group. Values with no standard error bars have n ≤ 2 per group. *significantly different from young SFA, $p \leq 0.05$.

2.4 Discussion

The primary findings from this study are as follows: 1) total eNOS protein content is not significantly altered with age throughout the arterial network; 2) CaM:eNOS interaction in young animals is heterogeneous throughout the arterial network; and 3) aging has no significant effect on Cav1:eNOS and CaM:eNOS interactions throughout the arterial network.

These findings emphasize the heterogeneous phenotype of the arteries and arterioles, especially from young animals, examined in this study. This heterogeneity exists primarily between the young SFA and large arteries (aorta, iliac, and femoral artery), muscular arteries (GFA), and arterioles from both oxidative and glycolytic skeletal muscles (WG3A, S2A). Even arterioles within an isolated skeletal muscle display a heterogeneous protein:protein interaction phenotype. These differences are likely due, at least in part, to the different anatomical structures, blood flow patterns, and metabolic milieu between oxidative and glycolytic skeletal muscles.

The effect that aging has on protein:protein interactions throughout the arterial tree are also varied. This likely reflects the tissue-specific effects of aging on blood flow patterns and the local metabolic environment. It is also possible that the age-related alterations in protein:protein interactions may confer some benefit in certain vessels that do not demonstrate age-related endothelial dysfunction. For example, Cav1 sequestering of eNOS may prevent eNOS from becoming a pro-oxidant enzyme in aged vessels that do not demonstrate impaired NO-mediated endothelial function (Karuppiah et al. 2011). Thus, it is conceivable that aging results in eNOS-associated protein:protein interactions

that are a mechanism to compensate for impaired NO bioavailability. However, the impact this increase in Cav1:eNOS interaction has on NO-mediated endothelial function remains to be determined, as no significant differences in Cav1:eNOS interactions were detected. However, it is possible that Cav1:eNOS interactions may be altered with age by mechanisms that would not be detected using co-immunoprecipitation and immunoblotting techniques (Aoki et al. 1999).

Interestingly, the CaM:eNOS interaction was only significantly affected by vessel heterogeneity in young animals when compared to young SFA. This may reflect heterogeneity of eNOS regulation by CaM throughout the arterial tree. Traditionally Cav1 and CaM have been thought to reciprocally regulate eNOS activity (Michel et al. 1997b; Michel et al. 1997c), while findings from the present study suggest that while this may be true, the protein:protein interactions do not show a pattern of reciprocal regulation (**Figuress**. **2.2, 2.3**).

Within the skeletal muscles of the hindlimb, the CaM:eNOS interaction was significantly higher in the young SFA compared to GFA and arterioles from both SFA and GFA. This may reflect the high level of control the SFA exerts over regulating blood flow to the primarily oxidative soleus muscle, both and rest and during aerobic exercise. However, further studies are needed to confirm these results and determine the role of CaM:eNOS interaction in NO-mediated endothelial function.

Results from this study demonstrate the heterogeneous phenotype of CaM:eNOS throughout the arterial network. The non-uniform effect of arterial structure and function on protein:protein interactions may contribute to the heterogeneous phenotype of

CaM:eNOS interaction. However, this study suggests that neither Cav1:eNOS nor CaM:eNOS interactions are altered with aging throughout the arterial network.

CHAPTER III

EFFECT OF AGE AND EXERCISE TRAINING ON PROTEIN:PROTEIN INTERACTIONS AMONG eNOS AND ITS REGULATORY PROTEINS IN RAT AORTAS*

3.1 Introduction

Aging is an independent risk factor for cardiovascular disease, as is impaired endothelial function (Celermajer et al. 1994b). Previous studies have demonstrated an age-related decline in endothelium-mediated vasorelaxation in aortas (Smith et al. 2006; Delp et al. 1995); however, the mechanism(s) accounting for the age-related decline in nitric oxide (NO)-mediated endothelial function in aged arteries is not fully understood. NO bioavailability is critical for maintaining normal endothelial function, and numerous studies have demonstrated reduced NO bioavailability in various vascular beds in older humans and aged animals (Muller-Delp et al. 2002b; Cernadas et al. 1998; Kang et al. 2009b; Celermajer et al. 1994b; van der Loo et al. 2000b; Trott et al. 2013; Trott et al. 2011; Woodman et al. 2003). Endothelial nitric oxide synthase (eNOS) is the enzyme responsible for generating NO in endothelial cells. In conduit arteries, age-related increases in eNOS content have been documented (van der Loo et al. 2000b; Cernadas et al. 1998); however, this finding has not been consistently reported by all investigators (Barton et al. 1997b). Post-translational modifications of the eNOS protein, independent of changes in eNOS protein content, may play a role in regulating eNOS activity and NO bioavailability in aged arteries (Smith and Hagen 2003; Michel et al. 1997b).

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Age-related changes in post-translational mechanisms like protein:protein interactions that modulate eNOS activity have not been thoroughly explored. eNOS activity is regulated by a number of proteins, including caveolin-1 (Cav1), a negative regulator of eNOS activity (Du and Chen 2009; Smith et al. 2006; Michel et al. 1997b), as well as heat shock protein 90 (Hsp90) and calmodulin (CaM), positive regulators of eNOS activity (Garcia-Cardena et al. 1998; Michel et al. 1997b). Evidence from *in vitro* studies suggests that eNOS interaction with Cav1 is increased with aging (Smith et al. 2006; Yoon et al. 2010) which may explain the reduced NO bioavailability seen in aged arteries. Additionally, there is evidence suggesting that aging reduces the interaction between eNOS and Hsp90 (Smith et al. 2006). Collectively, these studies identify key regulatory proteins of eNOS that may be of interest in elucidating a potential mechanism accounting for the impaired NO-mediated endothelial function with aging.

Aerobic exercise training has been shown to attenuate or reverse the detrimental effects of aging on endothelium-dependent dilation (DeSouza et al. 2000; Spier et al. 2004; Trott et al. 2009); however, the mechanism(s) accounting for the beneficial effect of exercise training is not fully understood. Shear stress has been identified as an important stimulus that initiates adaptations in the endothelium resulting in an increased NO bioavailability in aged vessels with aerobic exercise (Davis et al. 2001; Fisslthaler et al. 2000). Previous research has demonstrated that shear stress regulates Cav1 interactions with eNOS (Rizzo et al. 1998a), suggesting that Cav1:eNOS interaction may be a site of therapeutic interest in the prevention and treatment of cardiovascular diseases. Furthermore, endurance exercise training has been shown to enhance Hsp90

expression in soleus skeletal muscle tissue (Harris et al. 2008), which can potentially enhance NO bioavailability. When bound to eNOS, Hsp90 enhances eNOS enzyme activity and NO production. Importantly, the interactive effects of aging and exercise training on protein:protein interactions between Hsp90 and eNOS in arteries are unknown. CaM, a calcium-sensitive protein that displaces Cav1 from eNOS also facilitates NO production when bound to eNOS (Busse and Mülsch 1990). To date, little is known about the effects of age and exercise training on CaM and Cav1 protein content and its interaction with eNOS.

The purpose of the present study was to test the hypothesis that impaired endothelium-dependent relaxation in aged aorta is due to alterations in protein:protein interactions between eNOS and its regulatory proteins resulting in impaired NO-mediated relaxation. In addition, we hypothesized that endurance exercise training improves or restores NO-mediated vasorelaxation in aged aorta by reversing the detrimental effects of aging on protein:protein interaction between eNOS and its regulatory proteins.

3.2 Methods

3.2.1 Animals

Before the initiation of this study, approval was received from the University of Missouri and Texas A&M University Institutional Animal Care and Use Committees. Young (2 mo) and old (22 mo) male Fischer 344 rats were obtained from a commercial dealer (Harlan Sprague-Dawley, Indianapolis, IN) and housed at the University of Missouri College of Veterinary Medicine's Animal Care Facility or the Texas A&M

University Comparative Medicine Program Facility. The rats used in the present study were a subgroup of rats used in a previously published study of the effects of training on soleus muscle resistance arteries (Trott et al. 2009) . All rats were housed under a 12:12-h light-dark cycle and food and water were provided *ad libitum.* The rats were examined daily by the Animal Care Facility veterinarians. Fischer 344 rats were chosen for these experiments because of the absence of atherosclerosis and hypertension with age in this animal model (Lakatta 1995). Upon arrival, young and old rats were randomly assigned to either the sedentary (Sed) or exercise trained group (Ex) for a total of four groups of rats: *1)* young Sed (n = 10), *2)* young Ex (n = 10), *3)* old Sed (n = 10), and *4)* old Ex (n = 10).

3.2.2 Training protocol

The exercise training protocol used in this study has been described previously (Spier et al. 2004; Trott et al. 2009). In brief, rats were familiarized with running on a motorized treadmill and randomly assigned to an Ex or Sed group for 10-12 weeks. Rats in the Ex group ran 60 min/day, 5 days/week, at 15m/min (15° incline). Rats in the Sed group were restricted to their cages and did not exercise. To determine the efficacy of the endurance exercise training protocol, citrate synthase activity was measured in the vastus lateralis muscle (Srere 1969).

3.2.3 Isolation of aortas

Prior to removal of the aortas, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50-60 mg/kg body wt, ip). The thoracic and abdominal cavities were exposed and the abdominal aorta was dissected out and placed

in cold (4°C) MOPS buffered physiological saline solution (PSS), containing (in mM) 145.0 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.17 $MgSO_4$, 1.2 NaH_2PO_4, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA and 25.0 MOPS (pH 7.4). The aorta was then transferred to a Lucite chamber containing MOPS-PSS for removal of adipose and connective tissue, and sectioned into rings approximately 2 millimeters in axial length. Aortic rings used for functional analysis of vasoreactivity were mounted on a wire myograph. The remaining aortic segments were placed in 0.7 mL microcentrifuge tubes, snap frozen, and stored at -80°C for subsequent co-immunoprecipitation and immunoblot analysis.

3.2.4 Determination of vasorelaxation responses

The protocol used to determine aortic vasorelaxation responses has been described previously in detail (Delp et al. 1993). Briefly, aortic rings were mounted on two stainless steel wires, one attached to a force transducer, and the other attached to a micrometer microdrive. The aortic ring and myograph apparatus was placed in a Krebs bicarbonate buffer solution equilibrated at 37°C with 95% O_2-5% CO_2. Isometric tension was continuously monitored. Aortic rings were preconstricted with norepinephrine (10^{-7}M) and endothelium-dependent vasorelaxation responses were assessed in response to ACh stimulation by adding cumulative doses over the range of 10^{-10} – 10^{-4} M in half log increments as described previously (Delp et al. 1995). To determine the role of NO, ACh-induced vasorelaxation responses were assessed in the absence or presence of L-NG-nitroarginine methyl ester (L-NAME, 300μM) to inhibit NOS. Endothelium-independent relaxation responses to sodium nitroprusside (SNP) were measured by adding cumulative doses over the range of 10^{-10} – 10^{-4} M in half log increments.

3.2.5 Immunoprecipitation

To assess protein:protein interactions among eNOS and its regulatory proteins in aortas from young and old Sed and Ex rats, frozen aortic segments were homogenized using a mortar and pestle, then transferred to a microcentrifuge tube with a lysis buffer containing (in mM): 60.0 octyl-β-d-glucopyranoside (Sigma-Aldrich, O8001), 150.0 NaCl, 20.0Tris base, 1% protease inhibitor cocktail (EMD Millipore, 20-201), 1% phosphatase inhibitor cocktail (Sigma-Aldrich, P0044). The aortic segments were further homogenized using repeated freeze-thaw cycles with brief sonication. To collect the soluble proteins, the homogenate was centrifuged for 10 min at 12,000Xg at 4°C and the supernatant collected and used for co-immunoprecipitation. 20µL of Protein G/A agarose suspension was added to the lysate and incubated at 4°C for 1 hour under gentle rotary agitation. After centrifugation at 12,000Xg for 1 min, the supernatant was collected. 1 µg of polyclonal eNOS antibody was added to the lysate and incubated for 4 hours at 4°C under gentle rotary agitation. 15µL of Protein G/A agarose suspension was then added to the lysate and incubated for 1 hour at 4°C under gentle rotary agitation. The eNOS protein complex was then eluted from the lysate, washed three times and stored at -80°C in Laemmli buffer for immunoblotting.

3.2.6 Quantification of Cav1:eNOS, Hsp90:eNOS, and CaM:eNOS interactions

Relative differences in eNOS, Cav1, Hsp90, and CaM across age and training groups were assessed in aortas using immunoblot analysis as described in detail previously (Jasperse and Laughlin 1999). Briefly, protein content was assessed using a bicinchoninic acid protein assay. eNOS, Cav1, Hsp90, and CaM protein content was

evaluated using monoclonal antibodies (eNOS, Hsp90, and CaM, 1:1250; Cav1, 1:2500). Immunoblots were evaluated using enhanced chemiluminescence (ECL, Amersham) and densitometry using LAS-4000 Luminescent Image Analyzer and Multi-Gauge Image Analysis Software (FUJIFILM Medical Systems).

3.2.7 Statistical analysis

All values are presented as mean ± SE. Two-way ANOVA was used to determine between-group differences in body weight, citrate synthase activity, and protein:protein interactions. Concentration-response curves were analyzed by two-way ANOVA with repeated measures on one factor (dose) to determine whether vasorelaxation responses to ACh and SNP differed by group. Concentration-response data were expressed as a percentage of maximal possible vasorelaxation. Percent possible vasorelaxation was calculated as $[(D_{dose} - D_B)/(D_P - D_B)] \times 100$, where D_{dose} is measured tension for a given dose, D_B is baseline tension of an intervention, and D_P is maximal passive diameter. Statistical significance was set at the $P \leq 0.05$ probability level.

3.3 Results

3.3.1 Characteristics of rats

The body weights and citrate synthase activity of the rats used in this study have been published previously (Trott et al. 2009). Briefly, Old Sed rats were significantly heavier than Young Sed rats. Endurance exercise training significantly lowered body weight in Old Ex rats so they were not different from Young Sed or Young Ex rats. As reported previously (Trott et al. 2009), citrate synthase activity in the vastus lateralis was

significantly increased by training in both young (Young Sed: 29.9 ± 0.7 vs. Young Ex: 39.7 ± 3.7 μmol · min^{-1}·g wet wt $^{-1}$, $P \leq 0.05$) and old (Old Sed: 25.6 ± 1.8 vs. Old Ex: 38.7 ± 3.0 μmol · min^{-1}·g wet wt $^{-1}$, $P \leq 0.05$) rats, confirming the efficacy of the training protocol.

3.3.2 Effect of age and exercise training on endothelium-dependent and –independent vasorelaxation

3.3.2.1 ACh-induced relaxation

ACh-induced relaxation was significantly impaired in Old Sed aortas relative to Young Sed aortas **(Figure. 3.1A)**. Endurance exercise training significantly improved ACh-induced relaxation in old aortas such that ACh-induced relaxation was significantly greater in Old Ex than in Old Sed **(Figure. 3.1A)**. In addition, ACh-induced relaxation of Old Ex aortas was not different from that of Young Sed aortas **(Figure. 3.1A)**. Endurance exercise training also improved relaxation responses in aortas from young animals so that Young Ex was significantly greater than Young Sed **(Figure. 3.1A)**. All between-group differences in ACh-induced relaxation were abolished in the presence of L-NAME to inhibit NOS **(Figure. 3.1B)**.

3.3.2.2 SNP-induced relaxation

There were no significant between-group differences in vasorelaxation responses to SNP **(Figure. 3.2)**.

3.3.3 Effect of age and exercise training on eNOS associated protein:protein interactions

Total eNOS protein content was significantly greater in aortas from Old compared to Young rats **(Figure. 3.3)**. Cav1:eNOS interaction was significantly lower in aortas from Old Sed compared to Young Sed rats **(Figure. 3.4)**. Endurance exercise training did not alter Cav1:eNOS interactions in young or old aortas **(Figure. 3.4)**. Protein:protein interaction between Hsp90 and eNOS was not altered by age or exercise training **(Figure. 3.5)**. CaM:eNOS interaction was significantly lower in aortas from Old Sed compared to Young Sed **(Figure. 3.6)**; however, there was no effect of endurance training on CaM:eNOS interactions in young or old aortas.

Figure 3.1

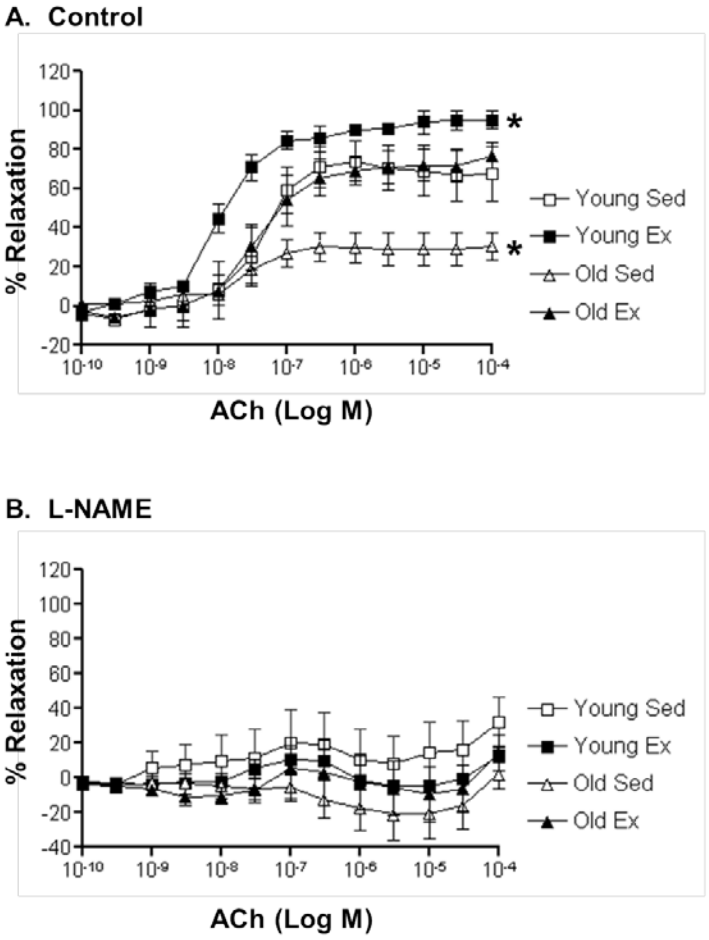

Figure 3.1. Acetylcholine (ACh)-induced relaxation in aortic rings in the absence (Panel A) or presence (Panel B) of L-NAME (300μM) to inhibit nitric oxide synthase. Sed, sedentary; Ex, exercise trained; Values are means ± SE; n = 10 per group. *Dose-response curve significantly different from all other curves, $p \leq 0.05$.

Figure 3.2

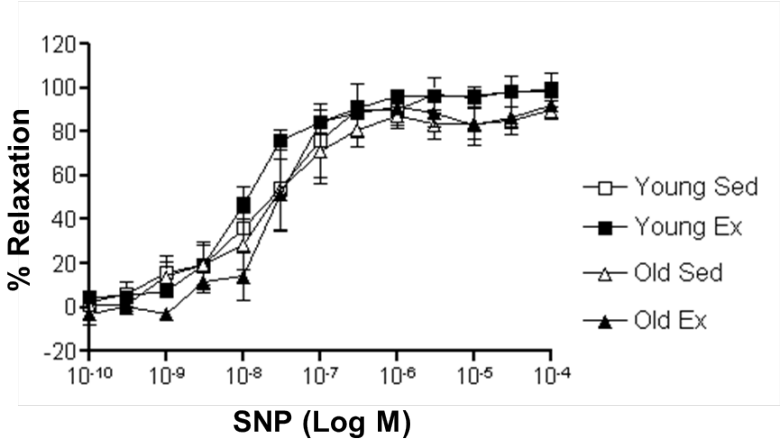

Figure 3.2. Sodium nitroprusside (SNP)-induced relaxation in aortic rings. Sed, sedentary; Ex, exercise trained; Values are means ± SE; n = 10 per group. Statistical analysis revealed no significant between-group differences.

Figure 3.3

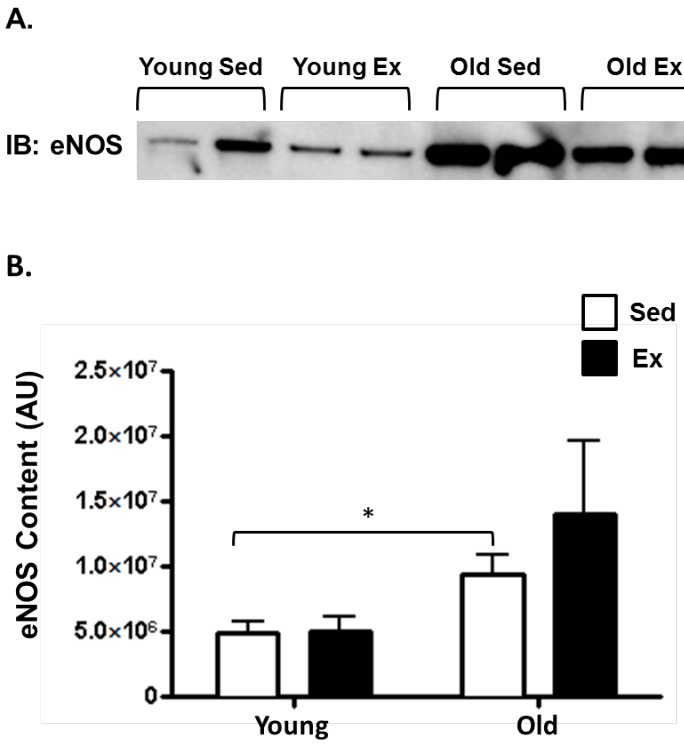

Figure 3.3. Total eNOS protein content in aortic rings. Sed, sedentary; Ex, exercise trained. Inset: representative immunoblot for eNOS. Values are means ± SE; n =6-10/group. *significantly different, $p \leq 0.05$.

Figure 3.4

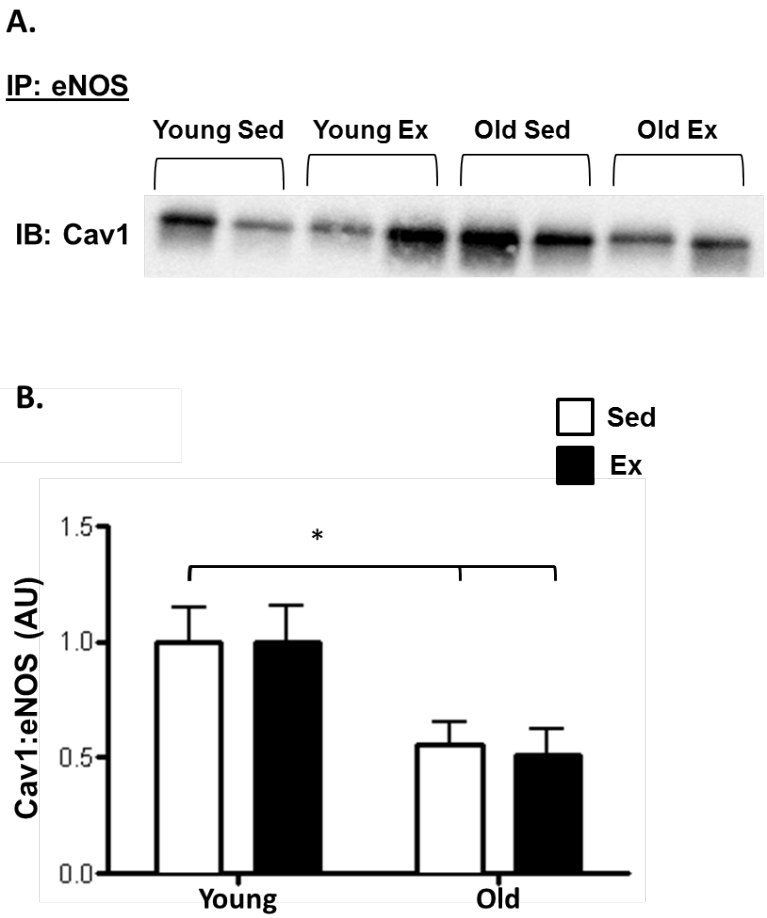

Figure 3.4. Cav1 association with eNOS. Antibodies against eNOS were used to immunoprecipitate eNOS protein. The immunoprecipitates were analyzed for associated Cav-1 by immunoblot. Sed, sedentary; Ex, exercise trained. Inset: representative immunoblot for Cav-1. Values are means ± SE. n = 6-10/group. *significantly different, $p \leq 0.05$.

Figure 3.5

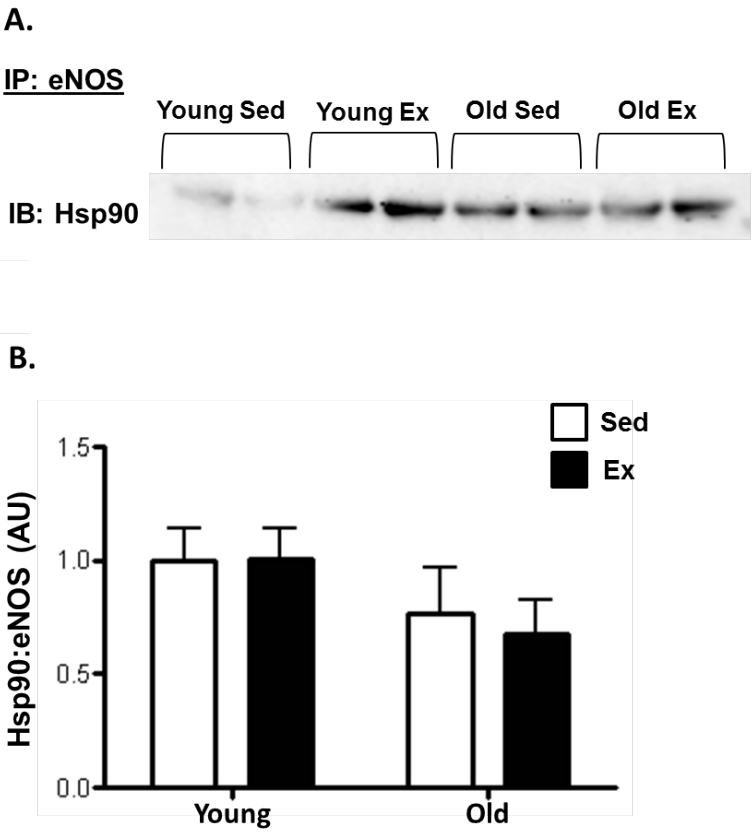

Figure 3.5. Hsp90 association with eNOS. Antibodies against eNOS were used to immunoprecipitate eNOS protein. The immunoprecipitates were analyzed for associated Hsp90 by immunoblot. Sed, sedentary; Ex, exercise trained. Inset: representative immunoblot for Hsp90. Values are means ± SE. n = 6-10/group. *significantly different, $p \leq 0.05$.

Figure 3.6

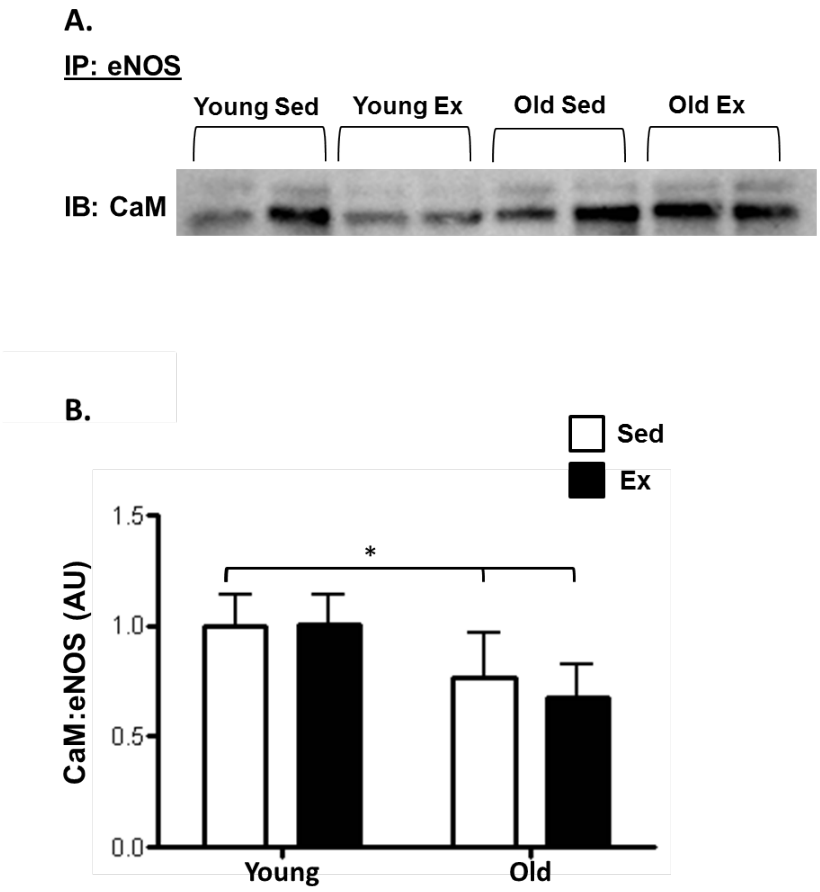

Figure 3.6. CaM association with eNOS. Antibodies against eNOS were used to immunoprecipitate eNOS protein. The immunoprecipitates were analyzed for associated CaM by immunoblot. Sed, sedentary; Ex, exercise trained. Inset: representative immunoblot for CaM. Values are means ± SE. n = 6-10/group. *significantly different, $p \leq 0.05$.

3.4 Discussion

The purpose of this study was to test the hypothesis that impaired endothelium-dependent relaxation in aged aorta is due to alterations in protein:protein interactions between eNOS and its regulatory proteins resulting in impaired NO-mediated relaxation. In addition, we hypothesized that endurance exercise training improves or restores NO-mediated vasorelaxation in aged aortas by reversing the detrimental effects of aging on protein:protein interaction between eNOS and its regulatory proteins. The primary novel findings of this study are that: 1*)* Aging resulted in reduced Cav1:eNOS and CaM:eNOS interactions, but had no impact on Hsp90:eNOS interactions, and 2) 10 weeks of endurance exercise training did not alter protein:protein interactions among eNOS and its key regulatory proteins. Additionally, we confirmed the presence of impaired NO-mediated, ACh-induced endothelium-dependent relaxation in Old Sed vs. Young Sed rat aortas, and demonstrated that 10 weeks of endurance exercise training completely restores NO-mediated, ACh-induced relaxation in aortas from old rats. Together, these results suggest that age-associated alterations in the interactions among eNOS and three of its key regulatory proteins may be a mechanism accounting for impaired NO-mediated vasorelaxation in aged arteries. Further, these results indicate that the beneficial effect of endurance exercise training in improving NO-mediated ACh-induced endothelial function with age is unlikely due to exercise-induced alterations in protein:protein interactions among eNOS and Cav1, Hsp90, or CaM.

Endurance exercise training has been shown to improve NO-mediated endothelium-dependent relaxation in aged conduit arteries (Delp et al. 1993) as well as

resistance sized arteries and arterioles (Spier et al. 2004; Trott et al. 2009). This in turn reduces the risk of developing cardiovascular diseases and helps maintain endurance exercise tolerance with age. However, the mechanism(s) accounting for this improvement is unclear. There is evidence to suggest that endurance exercise training increases eNOS protein content and mRNA expression in aortas, which may enhance NO production with aging (Tanabe et al. 2003b); however, increased eNOS expression is not always directly associated with increased NO bioavailability (Cernadas et al. 1998). Indeed, the interaction between eNOS and the regulatory proteins Cav1, Hsp90, and CaM play an integral role in regulating NO production by altering eNOS enzyme activity. Cav1, a negative allosteric regulator of eNOS activity, binds eNOS on its scaffolding domain and reduces eNOS enzyme activity (Ju et al. 1997; Michel et al. 1997b). When endothelial cells are stimulated with ACh, there is an increase in intracellular calcium which activates CaM, which then binds to eNOS causing Cav1 to dissociate from eNOS (Michel et al. 1997b). Additionally, Hsp90 binding to eNOS facilitates eNOS binding with CaM and dissociation from Cav1 (Brouet et al. 2001).

In the present study, aging decreased Cav1:eNOS interaction, which was contrary to our hypothesis that aging would result in an increase in the interaction between these proteins. Additionally, this finding differs from the findings of Smith et al (2006), who showed an increase in Cav1:eNOS interaction in aortic endothelial cells from old rats compared to young rats. It is important to note however that the rats used in our studies were Fischer 344 rats studied at 4 and 24 months, whereas rats used in the Smith et al. study were Fischer 344 x Brown Norway cross studied at 4 and 36 months.

Thus, strain and age differences may contribute to the differences in Cav1:eNOS data. Additionally, we showed a significant age-associated increase in total eNOS protein content, which could further affect the total ratio of Cav1 bound eNOS protein, while Smith et al showed no difference in total eNOS protein content between young and old aorta. Greater eNOS protein content in aged aortas would make detecting small differences in Cav1 binding more difficult.

Interestingly, Piech et al. (2003) showed reduced total Cav1 protein in aortas from spontaneously hypertensive rats at both 18 and 63 weeks of age compared to aged-matched Wistar-Kyoto rats. Similarly, Vera et al. (2007) also demonstrated reduced Cav1 protein content as well as elevated eNOS protein content in aortas from spontaneously hypertensive rats compared to age-matched Wistar-Kyoto rats. While neither study looked at the interaction between Cav1 and eNOS, it is conceivable that the reduced Cav1 protein content and increased eNOS protein content resulted in lower Cav1:eNOS interaction in hypertensive animals. Downregulation of Cav1 with hypertension would allow NO production to be maintained via elevated eNOS activity as a compensatory mechanism against the increase in oxidative stress found in hypertension (Piech et al. 2003). Hypertension has previously been described as accelerated aging resulting in similar impairments in vascular endothelial function between aging and hypertension (Taddei et al. 1997); therefore, it is possible that the reduced Cav1:eNOS interaction found in our study is a compensatory mechanism to help preserve eNOS enzyme activity and thus, NO production.

Results from the present study were consistent with our hypothesis that exercise training restores endothelium-dependent relaxation in aged aorta by enhancing NO-mediated relaxation; however, the beneficial effect of exercise training was not mediated by altered protein:protein interactions between eNOS and the three key regulatory proteins examined in this study. While this study focused on Cav1, CaM, and Hsp90, eNOS also interacts with other proteins in the endothelium, including Akt, Future studies examining the effects of age and endurance exercise training on other eNOS-associated proteins in rat aortas are certainly warranted. Additionally, there is evidence indicating that aging has a heterogeneous effect throughout the arterial tree on endothelial function. While we did not find any effect of exercise training on protein:protein interactions in aged aortas, it is possible that in smaller conduit or resistance-sized arteries, the effects of exercise training may alter protein:protein interactions between eNOS and Cav1, CaM, or Hsp90.

The mechanism accounting for the training-induced improvement in NO-mediated relaxation is not clear; however, it is conceivable that exercise training enhanced vascular antioxidant capacity in aged aortas. This speculation is supported by previous work by Trott et al (2009) indicating that exercise training increased extracellular superoxide dismutase protein (ecSOD) content and restored NO-mediated endothelium-dependent dilation in skeletal muscle resistance arteries. It is important to note that the aortas used in the present study were from the same animals used by Trott et al. (2009) to study skeletal muscle resistance arteries. Taken together, these results indicate that exercise training restores NO-mediated endothelial function in both conduit

and resistance arteries, which may reduce the risk of cardiovascular disease and improve exercise tolerance in the elderly.

In summary, the results of this study indicate that aging impairs NO-mediated, endothelium-dependent relaxation in aged aortas. Endurance exercise training reverses the detrimental effects of aging on endothelium-dependent relaxation in aortas by enhancing NO bioavailability. Contrary to our hypothesis, the enhancement in NO bioavailability was not mediated by exercise training-induced alterations in protein:protein interactions between eNOS and its regulatory proteins. Further study is needed to determine the mechanism accounting for the beneficial effect of exercise training on NO-mediated relaxation.

CHAPTER IV

SUMMARY AND CONCLUSIONS

4.1 Summary and conclusions

The purpose of this dissertation was to determine the effect of aging on NO-mediated endothelial function via protein:protein interactions among eNOS and its key regulatory proteins throughout the vascular tree and to determine the effect of exercise training on eNOS-associated protein:protein interactions with aging in the aorta.

The primary purpose of the first study was to examine the age-associated changes in Cav1:eNOS and CaM:eNOS interactions throughout the vascular tree, including large conduit arteries, small muscular arteries, and resistance-sized skeletal muscle arterioles. Given the heterogeneous nature of the arterial network, it was hypothesized that the protein:protein interactions examined would vary with vessel type, as would the effect of aging on these protein:protein interactions. Additionally, aging has been shown to have disparate effects on NO-mediated endothelial function in oxidative vs. glycolytic skeletal muscle feed artery and arterioles, further supporting the rationale for this study. This may contribute to the altered distribution of skeletal muscle blood flow from oxidative to glycoltic muscle, as well as impaired exercise tolerance seen with advanced age. The key findings of this study are: 1) there is heterogeneity of eNOS-associated protein:protein interaction phenotype throughout the arterial network; and 2) aging also has a heterogeneous effect on eNOS-associated protein:protein interactions throughout the arterial network.

While results of this study demonstrate the heterogeneous effect of aging on eNOS-associated protein:protein interactions through the arterial network, the implications of these alterations are currently unknown. It is likely that the metabolic milieu surrounding each branch of the arterial network interacts with the specific anatomy of that vessel to optimally regulate arterial tone throughout the arterial tree. This study focused on one mechanism as a potential mediator of age-associated impairments in NO-mediated endothelial function, but numerous mechanisms appear to contribute to the age-related decline in endothelial function. Age-related increases in oxidative stress have been shown to impaire NO-mediated endothelial function in soleus muscle feed arteries and arterioles, as well as in aortas. Further study is need to determine the contribution of both Cav1:eNOS and CaM:eNOS interactions to NO-mediated endothelial function in each vessel branch both in young healthy vessels and in aged vessels. A schematic outlining the key findings from this study is presented in **Figure 4.1**.

Figure 4.1

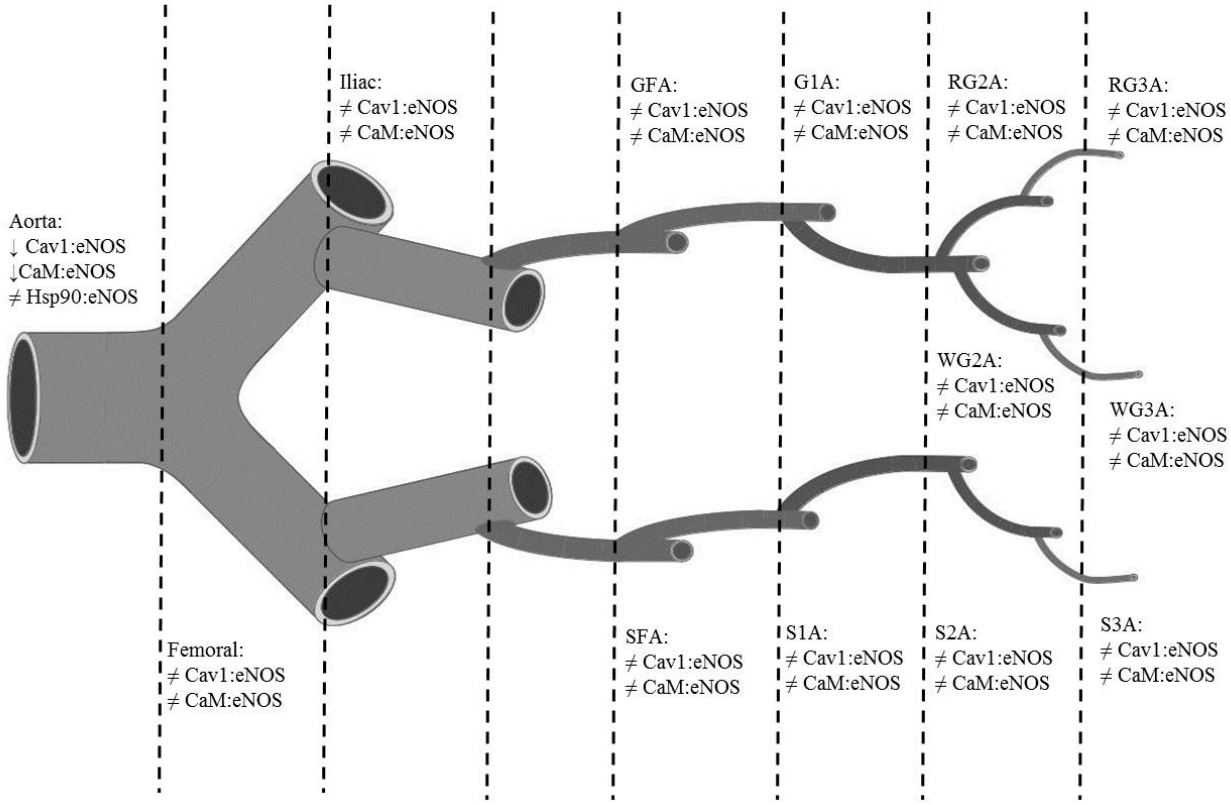

Figure 4.1. Schematic outlining changes in Cav1:eNOS and CaM:eNOS with aging. Key: ↓ interaction decreases with age; ≠ no significant change in interaction with age.

The primary purpose of the second study was to determine the effects of aging combined with endurance exercise training on NO-mediated endothelial function and Cav1:eNOS, Hsp90:eNOS, and CaM:eNOS interaction in rat aortas. Cav1 acts as a negative regulator of eNOS activity, thus increasing the amount of eNOS bound to Cav1 could potentially reduce NO bioavailability. Hsp90 and CaM are both positive regulators of eNOS when bound, and have to potential to enhance NO bioavailability. The hypotheses for this study were that aging would result in impaired NO-mediated vasorelaxation in aged aortas, and that this would be ameliorated with 10 weeks of endurance exercise training. Further, aging would increase Cav1:eNOS while reducing Hsp90:eNOS and CaM:eNOS interactions, with the effect of limiting NO bioavailability in aged aortas. Finally, endurance exercise training would reverse the detrimental effects of aging on protein:protein interactions among eNOS and its key regulatory proteins. The key findings of this study are that: 1) aging resulted in impaired ACh-induced endothelium-dependent relaxation in rat aortas; 2) 10 weeks of endurance exercise training completely restored ACh-induced relaxation in aortas from old rats, and; 3) All age and exercise effects were abolished in the presence of L-NAME to inhibit NOS; 4) Aging resulted in reduced Cav1:eNOS and CaM:eNOS interactions. These findings are consistent with our hypothesis that endurance exercise training mitigates the negative effects of aging on NO-mediated endothelial function. Additionally, aging also alters Cav1:eNOS and CaM:eNOS interactions, which may ultimately result in impaired NO bioavailability. While the decrease in Cav1:eNOS interaction with age found in this study appears to contradict previous work, it is possible that the dissociation of eNOS

from Cav1 is a compensatory mechanism against impaired NO bioavailability to increase eNOS enzyme activity. However, this compensation is not sufficient to restore NO bioavailability with age. This suggests that other mechanisms are also present that limit NO bioavailability. The finding that aging is associated with reduced CaM:eNOS interactions supports our hypothesis, and may be one mechanism that partially accounts for the decline in NO bioavailability with aging. Interestingly, we found no effect of exercise training on the three protein:protein interactions identified in our study. These results suggest that exercise training does not work through these protein:protein interactions to restore or maintain NO-mediated vasorelaxation in rat aortas, although this finding does not preclude the possibility that other protein:protein interactions regulating eNOS activity respond to exercise training. The overall mechanism outlined in this study is presented in **Figure 4.2.**

Figure 4.2

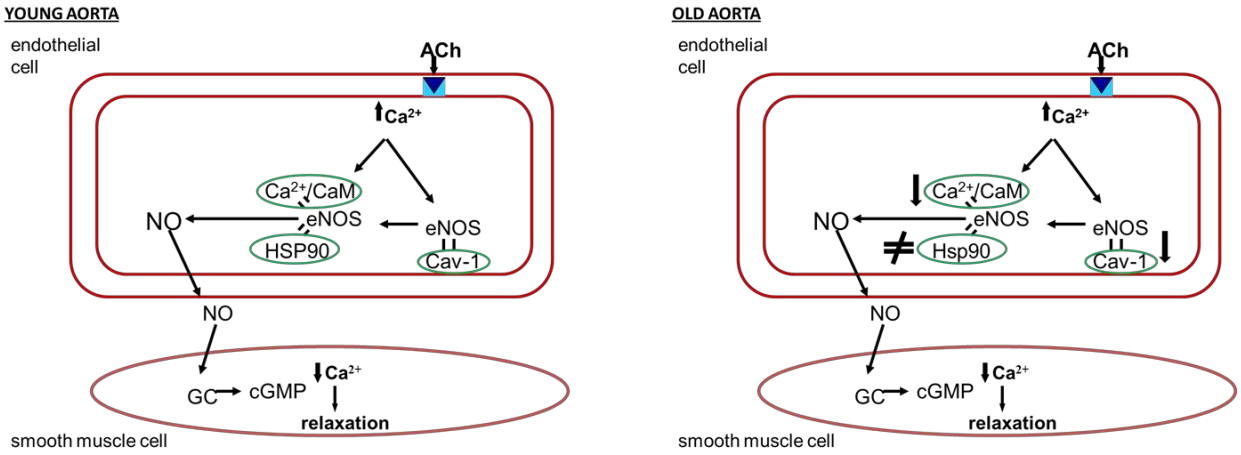

Figure 4.2. Proposed model of changes in Cav1:eNOS, CaM:eNOS, and Hsp90:eNOS with age and exercise training in rat aorta. Aging resulted in a decrease in Cav1:eNOS and CaM:eNOS interactions, but had no effect on Hsp90:eNOS interaction. 10 weeks of exercise training did not affect protein:protein interactions in either young or old aortas.

Collectively, these two studies highlight the heterogeneous nature of vascular aging throughout the arterial network. While the significance of the protein:protein interactions identified at each vessel is still a topic for further investigation, these studies are a first step in identifying a mechanism that has the potential to regulate NO bioavailability with aging. Additionally, the second study emphasizes the beneficial effect of endurance exercise training in restoring endothelial function and the role of key protein:protein interactions that may be involved in this process.

4.2 Limitations

There are some potential limitations to the studies presented in this dissertation that should be identified. First, these studies were not designed to mechanistically link the protein:protein interactions to NO bioavailability in the vessels we studied. While this remains and important link between the studies presented here and confirming the role of each protein:protein interaction, the biochemistry used in these studies required a significant amount of tissue which precluded the additional studies that would directly link changes in protein:protein interactions to changes in NO production. Alternatively, knockout animal models or siRNA studies would also yield important insights to the questions addressed in this dissertation; however, these methods are cost prohibitive.

Second, the protein:protein interactions studied in this dissertation are likely only a few of the protein:protein interactions that may contribute to regulating NO bioavailability. The interactions selected for this dissertation had significant support from the existing literature that they directly contribute to eNOS enzyme activity. These

interactions should provide a solid foundation for further exploration of this mechanism of eNOS regulation.

Finally, it should be noted that all protein:protein interactions were expressed as a ratio of the protein of interest to the immunoprecipitated eNOS. While this is an established method of presenting co-immunoprecipitation data, the age-related increase in eNOS protein content alone may alter the perceived protein:protein interactions.

4.3 Clinical relevance

The studies presented in this dissertation contribute to the development of therapeutic practices for older adults in preventing cardiovascular diseases that are prevalent with advanced age. The mechanisms accounting for age-related vascular pathologies appear to be specific to vessel type, and the first study in this dissertation systematically describes one mechanism in large conduit arteries, small muscular arteries, and arterioles. This has important clinical implications in that targeting a therapy to a specific vessel type would need to address the specific age-related changes in that vessel. Additionally, aerobic exercise has been established as a therapeutic strategy against the age-related decline in endothelial function, and the second study in this dissertation investigates a potential mechanism for preventing the age-related decline in endothelial function. While the findings in this study did not support our hypothesis, it is an important step in elucidating the mechanisms and pathways responsible for the protective effect of exercise training on vascular function.

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APPENDIX

RAW DATA TABLES

Table 1. Densitometry Values for eNOS blots (Figure 2.1)

Vessel	Number	Age	eNOS
Aorta	15874	Old	2828920
Aorta	15948	Old	1923284
Aorta	16077	Old	3569511
Aorta	16144	Old	3236856
Aorta	16219	Old	17928111
Aorta	16291	Old	4056471
Aorta	16365	Old	9856451
Aorta	15913	Young	2443182
Aorta	16039	Young	3172144
Aorta	16110	Young	3021445
Aorta	16182	Young	2133846
Aorta	16255	Young	3059072
Aorta	16329	Young	1516620
Aorta	16398	Young	1004033
Iliac	15805	Old	7970618
Iliac	15873	Old	5950400
Iliac	15947	Old	6175762
Iliac	16076	Old	6276924
Iliac	16143	Old	11948013
Iliac	16218	Old	6698636
Iliac	16289	Old	10668341
Iliac	16364	Old	13678472
Iliac	15775	Young	6773347
Iliac	15838	Young	6386039
Iliac	15912	Young	6222876
Iliac	16038	Young	9994104
Iliac	16109	Young	23277389
Iliac	16181	Young	15860946
Iliac	16254	Young	13695375
Iliac	16378	Young	16822028
Femoral	16363	Old	2867606
Femoral	16292	Old	2773075
Femoral	16217	Old	3539420
Femoral	16142	Old	4025345
Femoral	16075	Old	7357889
Femoral	15806	Old	6938630
Femoral	15872	Old	6433945

Table 1 continued

Vessel	Number	Age	eNOS
Femoral	16327	Young	2692977
Femoral	16253	Young	2482668
Femoral	16180	Young	2462662
Femoral	16108	Young	4630007
Femoral	15774	Young	7283229
Femoral	15837	Young	7756749
Femoral	16037	Young	7285422
Femoral	15911	Young	6846572
GFA	7	Old	12426416
GFA	5	Old	10450606
GFA	3	Old	7370194
GFA	1	Old	10328952
GFA	15	Old	7376028
GFA	13	Old	6643516
GFA	11	Old	5056720
GFA	9	Old	4863231
GFA	8	Young	11246731
GFA	6	Young	11274101
GFA	4	Young	8850318
GFA	2	Young	9581397
GFA	18	Young	4874365
GFA	16	Young	4845079
GFA	14	Young	5381467
GFA	10	Young	6775859
SFA	pooled	Old	5666621
SFA	pooled	Old	22139333
SFA	pooled	Old	8502373
SFA	pooled	Young	4902018
SFA	pooled	Young	8425816
SFA	pooled	Young	9569936
G1A	pooled	Old	5391149
G1A	pooled	Old	1569306
G1A	pooled	Young	5637504
G1A	pooled	Young	1586974
RG2A	pooled	Old	1762991
RG2A	pooled	Old	4526804
RG2A	pooled	Young	7398481
RG2A	pooled	Young	1733702
RG3A	pooled	Old	7860769
RG3A	pooled	Young	9215108

Table 1 continued

Vessel	Number	Age	eNOS
S1A	pooled	Old	2254486
S1A	pooled	Old	9133601
S1A	pooled	Old	14327350
S1A	pooled	Young	13521671
S1A	pooled	Young	11053543
S1A	pooled	Young	1584752
S2A	pooled	Old	7437848
S2A	pooled	Old	9269667
S2A	pooled	Young	8401487
S2A	pooled	Young	20368074
S3A	pooled	Old	6056566
S3A	pooled	Old	6966707
S3A	pooled	Young	5161176
S3A	pooled	Young	7188974
WG2A	pooled	Old	1676769
WG2A	pooled	Old	4410586
WG2A	pooled	Young	5820028
WG2A	pooled	Young	1628935
WG3A	pooled	Old	5501194
WG3A	pooled	Old	4824983
WG3A	pooled	Young	5727700
WG3A	pooled	Young	4213827
WG3A	pooled	Young	11618746

Table 2. Densitometry Values for eNOS and Cav1 (Figure 2.2)

Vessel	Number	Age	eNOS	Cav1
Aorta	15874	Old	2828920	2724901
Aorta	15948	Old	1923284	2628082
Aorta	16077	Old	3569511	2537707
Aorta	16144	Old	3236856	2434376
Aorta	16219	Old	17928111	8026812
Aorta	16291	Old	4056471	1401681
Aorta	16365	Old	9856451	3301940
Aorta	15913	Young	2443182	1318106
Aorta	16039	Young	3172144	5298554
Aorta	16110	Young	3021445	4468438
Aorta	16182	Young	2133846	1272840
Aorta	16255	Young	3059072	3754594
Aorta	16329	Young	1516620	1277201
Aorta	16398	Young	1004033	993433
Iliac	15805	Old	7970618	9006488
Iliac	15873	Old	5950400	7002914
Iliac	15947	Old	6175762	4947751
Iliac	16076	Old	6276924	5061395
Iliac	16143	Old	11948013	42642165
Iliac	16218	Old	6698636	37408155
Iliac	16289	Old	10668341	32935413
Iliac	16364	Old	13678472	31699221
Iliac	15775	Young	6773347	5710401
Iliac	15838	Young	6386039	5153780
Iliac	15912	Young	6222876	4953175
Iliac	16038	Young	9994104	4763558
Iliac	16109	Young	23277389	29464088
Iliac	16181	Young	15860946	35763658
Iliac	16254	Young	13695375	47782036
Iliac	16378	Young	16822028	34374906
Femoral	16363	Old	2867606	18343165
Femoral	16292	Old	2773075	14391302
Femoral	16217	Old	3539420	18206724
Femoral	16142	Old	4025345	20132448

Table 2 continued

Vessel	Number	Age	eNOS	Cav1
Femoral	16075	Old	7357889	9270321
Femoral	15806	Old	6938630	15309039
Femoral	15872	Old	6433945	11069955
Femoral	16327	Young	2692977	27419389
Femoral	16253	Young	2482668	25524759
Femoral	16180	Young	2462662	23293371
Femoral	16108	Young	4630007	17817320
Femoral	15774	Young	7283229	6203727
Femoral	15837	Young	7756749	8607567
Femoral	16037	Young	7285422	14637828
Femoral	15911	Young	6846572	13341706
GFA	7	Old	12426416	14625941
GFA	5	Old	10450606	9120073
GFA	3	Old	7370194	8511867
GFA	1	Old	10328952	11432762
GFA	15	Old	7376028	15540888
GFA	13	Old	6643516	26228304
GFA	11	Old	5056720	29890991
GFA	9	Old	4863231	28359450
GFA	8	Young	11246731	15301255
GFA	6	Young	11274101	18113111
GFA	4	Young	8850318	10352756
GFA	2	Young	9581397	19539012
GFA	18	Young	4874365	32206090
GFA	16	Young	4845079	22814729
GFA	14	Young	5381467	33998320
GFA	10	Young	6775859	18973901
SFA	pooled	Old	5666621	8473560
SFA	pooled	Old	22139333	9151252
SFA	pooled	Old	8502373	11368721
SFA	pooled	Young	4902018	7787865
SFA	pooled	Young	8425816	10175429
SFA	pooled	Young	9569936	11236060
G1A	pooled	Old	5391149	19172758
G1A	pooled	Old	1569306	4713403

Table 2 continued

Vessel	Number	Age	eNOS	Cav1
G1A	pooled	Young	5637504	22840103
G1A	pooled	Young	1586974	1846285
RG2A	pooled	Old	1762991	1860192
RG2A	pooled	Old	4526804	5315115
RG2A	pooled	Young	7398481	9145797
RG2A	pooled	Young	1733702	1829316
RG3A	pooled	Old	7860769	33688121
RG3A	pooled	Young	9215108	21754864
S1A	pooled	Old	2254486	5178818
S1A	pooled	Old	9133601	10141183
S1A	pooled	Old	14327350	9999012
S1A	pooled	Young	13521671	8626727
S1A	pooled	Young	11053543	11514302
S1A	pooled	Young	1584752	11007980
S2A	pooled	Old	7437848	25372234
S2A	pooled	Old	9269667	24451830
S2A	pooled	Young	8401487	20579520
S2A	pooled	Young	20368074	25863860
S3A	pooled	Old	6056566	30087603
S3A	pooled	Old	6966707	18447460
S3A	pooled	Young	5161176	27747941
S3A	pooled	Young	7188974	27438713
WG2A	pooled	Old	1676769	3161708
WG2A	pooled	Old	4410586	8634514
WG2A	pooled	Young	5820028	12657266
WG2A	pooled	Young	1628935	2166767
WG3A	pooled	Old	5501194	15311870
WG3A	pooled	Old	4824983	8793057
WG3A	pooled	Young	5727700	33830972
WG3A	pooled	Young	4213827	10358772
WG3A	pooled	Young	11618746	34089564

Table 3. Densitometry Values for eNOS and CaM (Figure 2.3)

Vessel	Number	Age	eNOS	CaM
Aorta	15874	Old	2828920	2125807
Aorta	15948	Old	1923284	2100519
Aorta	16077	Old	3569511	3910874
Aorta	16144	Old	3236856	3855000
Aorta	16291	Old	4056471	5142228
Aorta	16365	Old	9856451	3086545
Aorta	15913	Young	2443182	3024550
Aorta	16110	Young	3021445	5300122
Aorta	16182	Young	2133846	3485898
Aorta	16255	Young	3059072	4115113
Aorta	16329	Young	1516620	3945496
Aorta	16398	Young	1004033	1213614
Iliac	16143	Old	11948013	22456561
Iliac	16218	Old	6698636	27920996
Iliac	16289	Old	10668341	16647457
Iliac	16364	Old	13678472	18596692
Iliac	16109	Young	23277389	19182726
Iliac	16181	Young	15860946	21260530
Iliac	16254	Young	13695375	24494047
Iliac	16378	Young	16822028	23099979
Femoral	16363	Old	2867606	1930475
Femoral	16292	Old	2773075	1720049
Femoral	16217	Old	3539420	1834612
Femoral	16142	Old	4025345	1536532
Femoral	16075	Old	7357889	53682099
Femoral	15806	Old	6938630	63942643
Femoral	15872	Young	6433945	51135785
Femoral	16327	Young	2692977	2170651
Femoral	16253	Young	2482668	2031393
Femoral	16180	Young	2462662	1706425
Femoral	16108	Young	4630007	1694377
Femoral	15774	Young	7283229	31361392
Femoral	15837	Young	7756749	49691139
Femoral	16037	Young	7285422	67135353
Femoral	15911	Old	6846572	53058669

Table 3 continued

Vessel	Number	Age	eNOS	CaM
GFA	7	Old	12426416	12747217
GFA	5	Old	10450606	9862242
GFA	3	Old	7370194	8996341
GFA	1	Old	10328952	8785893
GFA	15	Old	7376028	22188254
GFA	13	Old	6643516	53280851
GFA	11	Young	5056720	46107120
GFA	9	Young	4863231	34361961
GFA	8	Young	11246731	14138121
GFA	6	Young	11274101	20810655
GFA	4	Young	8850318	9736387
GFA	2	Young	9581397	19028701
GFA	18	Young	4874365	44480277
GFA	16	Young	4845079	19263151
GFA	14	Old	5381467	22595499
GFA	10	Old	6775859	21576540
SFA	pooled	Old	5666621	90658644
SFA	pooled	Old	22139333	73301037
SFA	pooled	Old	8502373	83098934
SFA	pooled	Old	4902018	72517932
SFA	pooled	Old	8425816	100124821
SFA	pooled	Old	9569936	82743612
G1A	pooled	Young	5391149	5776373
G1A	pooled	Young	1569306	14194830
G1A	pooled	Young	5637504	7797078
G1A	pooled	Young	1586974	11632034
RG2A	pooled	Young	1762991	3733297
RG2A	pooled	Young	1733702	3636034
RG3A	pooled	Old	7860769	63189084
RG3A	pooled	Old	9215108	35977085
S1A	pooled	Old	2254486	32054805
S1A	pooled	Old	1584752	11572378
S2A	pooled	Young	7437848	6206463
S2A	pooled	Young	9269667	36976506
S2A	pooled	Old	8401487	5563065

Table 3 continued

Vessel	Number	Age	eNOS	CaM
S2A	pooled	Old	20368074	44311017
S3A	pooled	Young	6056566	6326281
S3A	pooled	Young	6966707	52039590
S3A	pooled	Old	5161176	7277516
S3A	pooled	Young	7188974	56831175
WG2A	pooled	Old	1676769	10121292
WG2A	pooled	Young	1628935	6228604
WG3A	pooled	Young	5501194	31709004
WG3A	pooled	Old	5727700	8961357
WG3A	pooled	Young	11618746	55929091

Table 4. Densitometry Values for eNOS blots (Figure 3.3)

Animal	Vessel Number	Group	eNOS
192	23692	Ysed	11086467
196	23789	Ysed	6861357
197	averaged	Ysed	3961956.5
198	averaged	Ysed	3737037.5
201	23903	Ysed	4137772
222	averaged	Ysed	2235769.5
223	averaged	Ysed	2687555
224	24395	Ysed	3494156
228	24474	Ysed	5378254
199	23866	Yex	5916685
200	23889	Yex	4107935
202	averaged	Yex	13145313.5
203	averaged	Yex	5535622.5
209	averaged	Yex	2842291
210	24113	Yex	2244742
218	24280	Yex	4510037
221	24336	Yex	3535956
225	24417	Yex	3679454
187	23601	Osed	11822782
188	23617	Osed	11402838
193	23731	Osed	14271160
194	23750	Osed	4287122
205	averaged	Osed	8244826
207	averaged	Osed	2376102
208	24082	Osed	8020135
213	24186	Osed	14527918
226	24435	Osed	3932943
227	24455	Osed	15119998
211	24142	Oex	40561747
212	averaged	Oex	5962293
214	averaged	Oex	11278278
215	averaged	Oex	15980545.33
216	averaged	Oex	5778732
217	averaged	Oex	4612531.667

Table 5. Densitometry Values for eNOS and Cav1 (Figure 3.4)

Animal	Vessel	Group	eNOS intensity	Cav1 intensity
192	23692	Ysed	11086467	0.30576621
196	23789	Ysed	6861357	0.510242362
197	averaged	Ysed	3961956.5	1.359136671
198	averaged	Ysed	3737037.5	1.153844253
201	23903	Ysed	4137772	0.57324594
222	averaged	Ysed	2235769.5	1.124300323
223	averaged	Ysed	2687555	0.955492204
224	24395	Ysed	3494156	1.692102184
228	24474	Ysed	5378254	1.312982801
199	23866	Yex	5916685	0.628006392
200	23889	Yex	4107935	0.842806179
202	averaged	Yex	13145313.5	0.53769847
203	averaged	Yex	5535622.5	0.978038749
209	averaged	Yex	2842291	0.953043408
210	24113	Yex	2244742	1.092084079
218	24280	Yex	4510037	0.498074406
221	24336	Yex	3535956	2.021260163
225	24417	Yex	3679454	1.410382356
187	23601	Osed	11822782	0.310732026
188	23617	Osed	11402838	0.297441479
193	23731	Osed	14271160	0.328127076
194	23750	Osed	4287122	0.921074091
205	averaged	Osed	8244826	0.636914073
207	averaged	Osed	2376102	1.164842819
208	24082	Osed	8020135	0.299970013
213	24186	Osed	14527918	0.429327175
226	24435	Osed	3932943	0.664613751
227	24455	Osed	15119998	0.537428775
211	24142	Oex	40561747	0.109384761
212	averaged	Oex	5962293	0.513639796
214	averaged	Oex	11278278	0.431452868
215	averaged	Oex	15980545.33	0.341190042
216	averaged	Oex	5778732	0.746789539
217	averaged	Oex	4612531.667	0.911300599

Table 6. Densitometry Values for eNOS and Hsp90 (Figure 3.5)

Animal	Vessel	Group	eNOS	Hsp90
197	23807	Ysed	2649704	2243425
198	23846	Ysed	2705715	2219985
201	23903	Ysed	4137772	2387207
223	24379	Ysed	3984216	2308077
224	24395	Ysed	3494156	1602664
228	24474	Ysed	5378254	1604505
202	23922	Yex	8581855	6002440
203	23942	Yex	2254479	1969533
218	24280	Yex	4510037	2452282
221	24336	Yex	3535956	1551894
225	24417	Yex	3679454	1610468
193	23731	Osed	14271160	11118482
194	23750	Osed	4287122	3168677
208	24082	Osed	8020135	2623391
213	24186	Osed	14527918	1727029
226	24435	Osed	3932943	2511840
227	24455	Osed	15119998	1970311
212	24163	Oex	5609000	2434281
214	24202	Oex	11066434	7644666
215	averaged	Oex	13811122	6302875
216	24244	Oex	7403776	1695894
217	24251	Oex	7736602	1573572

Table 7. Densitometry Values for eNOS and CaM (Figure 3.6)

Animal	Vessel	Group	eNOS	CaM
192	23692	Ysed	11086467	2368471
196	23789	Ysed	6861357	2009716
224	24395	Ysed	3494156	3363603
228	24474	Ysed	5378254	3492440
199	23866	Yex	5916685	1975547
200	23889	Yex	4107935	1949793
221	24336	Yex	3535956	3855570
225	24417	Yex	3679454	3240862
186	23601	Osed	11822782	2562151
197	23617	Osed	11402838	2084239
213	24186	Osed	14527918	2632914
227	24455	Osed	15119998	3334562
211	24142	Oex	40561747	2511489
212	24161	Oex	6315586	2355425
216	24244	Oex	7403776	3116903
217	24251	Oex	7736602	2725558